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# STUDIES IN REPERFUSION INJURY AND ITS ROLE IN THE IMMEDIATE GRAFT FUNCTION FOLLOWING LIVER TRANSPLANTATION

by

KHALID.I. BZEIZI.

*Submitted for the degree of Doctor of Medicine to the University of Edinburgh*

*1998*

# DECLARATION

I declare that this thesis has been composed by me and that the work contained within it was performed by me except where clearly stated otherwise. The entire work was performed while I held a post at the Department of Medicine and the Centre for Liver & Digestive Diseases, and Scottish Liver Transplant Unit at the Royal Infirmary of Edinburgh. I have not submitted this thesis for any other professional qualification.

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Professor Hayes encouraged me to consider my research in the area of reperfusion injury in liver transplantation, helped me to refine and conduct my research protocols and engaged me in many discussions about the contents of this thesis. This work has been truly educational and his involvement in all aspects of the production of this thesis is deeply appreciated.

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My family has been of tremendous support in conducting this research. My wife Jumana in particular has been instrumental with her constant encouragement and understanding in making it possible to have this work finished. I am deeply grateful to her.

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## Publications :

1. KI. Bzeizi, A. Soutar, PC. Hayes. Free radical production following vascular reperfusion. The Lancet, 1993; 342: 61-62 (letter).

2. KI. Bzeizi, R. Dawkes, NJF. Dodd, J. Plevris, PC. Hayes. Graft dysfunction following liver transplantation: Role of free radicals. Journal of Hepatology, 1997; 26(1): 69-74.

3. KI. Bzeizi, R. Jalan, I. MacGregor, O. Drummond, A. Lee, PC. Hayes. Neutrophil Elastase: A determinant of endothelial damage and reperfusion injury in liver transplantation. Transplantation, 1996; 62: 916-920.

4. KI. Bzeizi, R. Jalan, N. Henderson, A. Lee, PC. Hayes. Influence of cGMP changes on the haemodynamics following reperfusion in liver transplantation. Transplantation, 1997; 63(3): 403-406.

5. KI Bzeizi, R. Jalan, J. Plevris, PC. Hayes. Primary Graft Dysfunction: From pathogenesis to prevention. Liver Transplantation & Surgery, 1997; 3(2): 137-148.

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**ABSTRACTS:**

1. Kl. Bzeizi, I. MacGregor, R. Jalan, O. Drummond, A. Lee, PC. Hayes. Role of neutrophil activation in reperfusion injury following liver transplantation. Gut, 1994; 35(2): S10.

2. Kl. Bzeizi, R. Dawkes, I Armstrong, PC. Hayes. Role of reactive oxygen intermediates in the pathogenesis of primary graft dysfunction following OLT. Gut, 1994; 35: S70.

3. J.Plevris, K. Bzeizi, R.Jalan, A. Lee, NDC. Finlayson, PC. Hayes. Gratft dysfunction following liver transplantation is determined by the degree of reperfusion injury and predicted by ICG clearance. Hepatology, 1995; 22(4): 138A.

4. Kl. Bzeizi, I. MacGregor, R. Jalan, O. Drummond, A. Lee, PC. Hayes. Endothelial injury following liver graft reperfusion, and the role of neutrophils. Gastroenterology, 1996; 110(4): A1160.

5. JN.Plevris, Kl. Bzeizi, R. Jalan, M.Dollinger, A.Lee, NDC. Finlayson, PC. Hayes. Indocyanine Green clearance reflects the degree of reperfusion injury and accurately predicts graft function following liver transplantation. Gut, 1995; 37(1): F189.

6. Kl. Bzeizi, N. Henderson, J. Dillon, A. Lee, PC. Hayes. Role of cGMP on the haemodynamic changes during liver transplantation. Gut, 1995; 37(1): F191.

This thesis has provided evidence of increased ROIs activity following reperfusion of liver grafts. The magnitude of changes correlated with the severity of graft dysfunction. It also demonstrated that neutrophil activation occurs following graft reperfusion with evidence of endothelial cell damage as a result of such activity.

This thesis has shown that an ICG clearance <200ml/min measured within 24 hrs following liver transplantation predicted graft dysfunction due to reperfusion injury with high specificity and sensitivity.

Finally this thesis has demonstrated that the haemodynamic changes following reperfusion of liver graft during transplantation involve predominantly the pulmonary vasculature. It also demonstrates that these changes correlated significantly with a reduction in the levels of cGMP, which suggests a causal relationship. The endothelins levels in blood were raised before transplantation and remained so post-reperfusion, with no evidence of a role to play in the haemodynamic changes during transplantation.

# CHAPTER ONE-- INTRODUCTION

INTRODUCTION

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# INTRODUCTION

The liver graft often fails to function adequately after transplant operation for no clear reason. This thesis explores the humoral changes during the reperfusion phase of the transplant operation that could have a profound effect on graft outcome. The reperfusion phase of the operation is a critical stage when haemodynamic instabilities may occur with unfavourable consequences. Numerous studies on animal models and also in human organ transplantation (e.g. heart, kidneys and lungs) suggested development of ischaemia-reperfusion injury that may lead to graft failure. This chapter serves as a background for this thesis. The history of liver transplantation is briefly reviewed, followed by a detailed account of liver graft dysfunction including the clinical features and the potential pathogenic mechanisms. Aspects of preservation fluids including the rationale behind their developments and the nature of their contents are discussed in detail. The features of cold ischaemia are discussed followed by detailed account on the changes during reperfusion. Finally, the hypotheses behind the research in this thesis and a brief description of the studies are included at the end of this chapter.

## Historical Background

The first report of a liver transplantation was by Stuart Welch in (1955). It was a description of auxiliary canine liver transplantation that involved insertion of an extra liver into the pelvis or right paravertebral gutter of non-immunosuppressed recipients. Cannon (1956) speculated that because the liver played an important role in rejection, it might be the main factor for the rapid destruction of the grafts reported following auxiliary transplantation. He was the first to describe orthotopic liver transplantation (OLT) (i.e. replacement of the liver in its original site), but again as the case with the auxiliary technique, none of his dogs survived the operation.

Further attempts with OLT were described by other workers, and despite the absence of effective immunosuppression at that time, a solid basis for future work with OLT was laid throughout that period. In 1960, Moore reported on 31 canine experiments with 7 survivors of 4-12 days. Rejection was present after 5-6 days and was the main cause of death.

From the early stages of liver transplant history, the importance of graft preservation was appreciated and to start with, intra-portal infusion of chilled electrolyte solutions were used in the same way as is practised clinically today. Improved infusates in the succeeding years eventually

replaced the lactate Ringer's and saline solutions that were used originally. Use of University of Wisconsin (UW) solution in 1987, dramatically increased the length of safe preservation time from about 5 hours to 18-24 hours.

Venous bypass was used in canine liver transplantation as early as 1960 by Moore. It passively redirected blood from the occluded splanchnic and systemic venous beds to the superior vena cava during the anhepatic stage while recipient hepatectomy was performed and the new liver installed. Although venous bypass has not always been essential, it however made the operation less stressful in humans and played a role in further expansion of the transplant service.

The clinical trials of human liver transplantation started in 1963. Seven liver transplantations were reported by three different centres (Starzl, 1996). The results were disappointing and attempts for further transplantation stopped for a few more years up until 1967 when polyclonal antilymphocyte globulins were added to the standard immunosuppression of steroids and azathioprine.

The longest survival of liver transplant recipients treated between July 1967 and March 1968 was 30 months (Starzl 1996). For the next 12 years, the 1 year mortality rate after liver transplantation never fell below 50%.

The losses were concentrated in the first postoperative months; after this initial period the life survival curve flattened, leaving a residual group of stable and remarkably healthy survivors.

Introduction of cyclosporin to clinical use in 1979 (Calne et al), had a remarkable impact on the success of organ transplantation. Of the first 12 liver recipients treated with cyclosporine and prednisolone in the first 8 months of 1980, 11 lived for more than a year (Starzl et al ) and 7 were still alive over 12 years later. As the news was confirmed that a 1 year patient survival rate of at least 70% was readily achievable, new liver programs proliferated worldwide.

Although the progress of liver transplantation was dominated by improvements in immunosuppression, there were other significant developments, including modifications in the details of the operation itself. The incidence of biliary duct complications has significantly decreased. Management of coagulopathies was facilitated by the use of the thromboelastogram to follow the minute-to-minute clotting changes in the operating room (Kang et al, 1985). The systematic use of veno-venous bypasses without anticoagulation also greatly diminished the occurrence of life thretening haemorrhages common at one time

## GRAFT DYSFUNCTION

Even with current optimal clinical liver preservation strategies, including short preservation time, some degree of clinical and biochemical dysfunction almost invariably occurs, the severity of which correlates with the degree of hepatic injury. Shaw et al (1985), were probably the first to use the term Primary non-function (PNF) to describe early graft failure post-transplantation. Many define PNF loosely as "initial poor hepatic function" (Greig et al 1989) or immediate graft failure (D'Alessandro et al 1993). Almost all omit any reference to a specific time course, although most would agree that PNF includes graft failure occurring from within hours to the first few days after transplantation. Differences in definition arise because organ failure exists as a continuum from potentially reversible dysfunction, namely primary graft dysfunction (PGD) to complete and unequivocal failure. PNF is considered to be a diagnosis of exclusion (Kakizoe et al 1990) and it represents the failure of an allograft soon after revascularization with no obvious cause, leading either to retransplantation or to the patient's death.

The reported incidence of PNF varied from 2-10% according to the criteria used for its definition (Greig et al 1989, Kamath et al 1990). It is generally, the most common cause of early graft loss, accounting for up to 40% of

such failures (D'Alessandro et al, 1992), and, has been the commonest indication for retransplantation within the first weeks following OLT (Howard et al 1990, Clavien et al 1992). There is however, a general feeling that the PNF could be significantly underreported. Deaths occuring early in the post-transplant period and attributed to sepsis, irreversible neurological injury, multiple organ system failure, and other nonhepatic causes may actually be the indirect effects of a non-functioning graft (Kakizoe et al, 1990).

Primary graft dysfunction (PGD) is a term covers the whole spectrum of graft failure with PNF representing its extreme and severe form. Even, in its mild form, PGD could result in slow postoperative recovery, prolonged intensive care stay (Ploeg et al, 1993), and subsequent allograft rejection (Howard et al 1990).

Patients with PNF develop encephalopathy, haemodynamic instability, and produce scanty, and pale bile. PNF is associated with increasing glucose requirement, coagulopathy, markedly raised plasma transaminases, metabolic acidosis and renal failure. Histologically the appearance ranges from mild cellular infiltration to hepatocyte ballooning with focal or zonal necrosis, along with microvesicular steatosis (Ludwig, 1988). The ultrastructural findings are classically seen in the sinusoidal lining cells (SLC), and include endothelial cell vaculisation, mitochondrialysis, and

plasma membrane interruption with poor rounding of cells (Momi et al, 1989).

Considerable advances have been made in our understanding of the pathogenesis of graft dysfunction following OLT. The following is a review of the literature concerning the potential mechanisms in the aetiology of graft dysfunction.

## GRAFT DYSFUNCTION: PATHOGENESIS

In most of the cases, PGD is likely to be the product of a number of different factors that arise either during and/or after transplantation. Such factors include: donor status; organ preservation; and reperfusion. By definition, PGD does not include the technical difficulties that can lead to graft failure. The following section deals with the perceived relative importance of these factors in the pathogenesis of PGD.

## DONOR FACTORS:

Although PGD has been described in organs retrieved from "perfect" donors, there has been a general acceptance that it occurs with greater frequency in "marginal" donors. The decision regarding graft suitability at the time of retrieval is often difficult. The time available is usually limited for any detailed objective tests to be performed in those circumstances. Potential donors with ongoing liver disease, abnormal liver functions, positive viral hepatitis screen, or known with alcohol or drug abuse are

usually excluded. The macroscopic appearance of the liver at the time of retrieval may be a helpful albeit late guide in determining graft suitability.

Donor issues which have received particular interest as are thought to be of potential influence of graft viability, included fatty changes of the liver, donor age, nutrition, and haemodynemic instability.

***Hepatic Steatosis:***

Fatty infiltration of the donor liver has been suggested as a risk factor for graft dysfunction and is frequently related to alcohol abuse (Strasberg et al 1994, Gao et al 1995). Grafts from malnourished, obese or older donors may also show fatty infiltration. The prevalence of fatty liver among the potential donor liver population is high, ranging from 13-26% (D'Alessandro et al 1991, Markin et al 1993). A key problem to the issue of transplantation of fatty liver is to establish the degree of steatosis that makes a donor organ unacceptable (Trevisani et al, 1996). Fatty liver is classified as mild if up to 30% of hepatocytes are steatotic, moderate when 30-60% of cells are involved, and severe if the figure is >60%. The latter has been identified as an absolute risk factor for graft failure and such grafts should be excluded. Adam et al (1991) found no difference in the one month survival of grafts with mild to moderate steatosis when compared with non-steatotic organs. These findings however were not

supported by Ploeg et al (1993) who reported using multivariate analysis that moderate steatosis was an independent risk factor for initial poor graft function

The mechanism by which fatty infiltration causes graft failure is unclear. A mechanical disruption of the hepatic sinusoids is thought by many to be the principle cause of graft dysfunction. Todo et al (1989) postulated that post-reperfusion, ruptured hepatocytes release fat contents into hepatic sinusoids which then coalesce into very large fat droplets which compress and obstruct the hepatic microcirculation. This leads to hepatocyte damage by local ischaemic effect. Release of free lipid from the damaged fat-laden hepatocyte into the sinusoidal circulation provides a substrate for subsequent lipid peroxidation by reactive oxygen intermediates during reperfusion and results in progressive endothelial cell injury that further impairs the hepatic microcirculation and subsequent liver graft function (Teramoto et al 1993). Enhanced Kupffer cell activity in grafts with marked fatty infiltration was noted, and this has been suggested as another important pathogenic mechanism (Teramoto et al 1993). Kupffer cells as will be discussed later are major sources of reactive oxygen intermediates, and their released cytokines have a potential role in reperfusion injury and graft dysfunction.

***Nutritional status:***

The effect of donor nutritional status on subsequent allograft function remains unclear. Prolonged fasting has been shown to result in severe hepatocyte deglycogenation, which compromises ATP production in the post-reperfusion period and potentially increases the susceptibility of the liver to ischaemia-reperfusion injury (Morgan et al 1991, Boudjema et al 1990). Sankary et al (1992) however, have demonstrated on animal studies improved graft function in response to fasting. They speculated that the non-parynchymal cells such as Kupffer cells which potentially contribute to reperfusion injury are inactivated by fasting more than the hepatocytes. So although fasting may deplete parenchymal cells of their energy source causing biochemical abnormalities, but improved whole organ viability suggests that this effect may be overridden by a positive effect on non-parencymal cells.

***Donor age:***

Studies assessing the role of donor age on graft function have shown variable results. Whilst Ploeg et al (1993) identified age as a risk factor using multivariate analysis This was not found in a larger group of patients reported by Adam et al (1991). Hoofnagle et al (1996), in a large series concluded that receipt of an older donor liver adversely affects the outcome of liver transplantation. Three-month graft survival was 10% less (81% vs. 91%) for recipients of livers from donors 50 years of age or older

than recipients of livers from younger donors. The study also showed a higher retransplantation rate among recipients of older livers, mainly during the first 3 months after transplantation. The differences in survival could not be attributed to differences in recipient population, such as the recipient's age, severity of liver disease, or diagnosis. In addition, the differences could not be attributed to other features of the donors, such as sex, cause of death, time in the hospital, or time in ICU before donation. The use of older donor livers was also associated with manifestations of poor initial graft function, although not entirley with PNF. The poor graft function might then have predisposed the transplantation recipient to multiple complications such as rejection and opportunistic infections.

Although the mechanisms underlying the poor initial graft function by older donor livers remain unclear, it has been noted that elderly donors often demonstrate steatosis which might have a role to play in the onset of poor graft function.

**Haemodynamic Status of the Donor**

This is thought to influence graft function after transplantation. Hypotensive or hypoxic episodes in a donor could adversely affect the liver especially with the added insult provided by the preservation process. Large doses of vasopressors such as adrenaline, or vasopressin may significantly contribute to the iscahemic injury. None of the studies

however looking into parameters such as hypotension, cardiac arrest or hypoxia of the donor demonstrated an increased incidence of poor graft function in presence of the above factors (Makowka et al 1987, Greig et al 1990). In an extreme situation, the human liver has been found to tolerate at least 1 hour of warm ischaemia, and excellent immediate function has been shown in organs obtained from non-heartbeating donors with warm ischaemia times of up to 45 minutes. Moreover, the incidence of PNF in this obviously suboptimal donor group appears to be the same as that pre-retrieved under more controlled conditions (Ericzon et al 1987).

## PRESERVATION

Organ preservation is a key issue in the success of organ transplantation, Organ preservation technology allows the sharing of cadaveric organs between hospitals of relatively great distances, thus giving more patients with end-stage organ disease an opportunity to receive an organ transplant. Currently, methods to preserve the liver appear adequate to meet most clinical needs, particularly that organs be preserved for a period of about 24 hours (Southard et al 1996).

Clinical cold storage of the kidney was begun by Collins et al (1969) with the development of Collins' solution (a phosphate-buffered solution containing a high concentration of glucose). Belzer and Southard developed the University of Wisconsin (UW) cold-storage solution (table 1.1), which has been effective for simple cold storage of solid organs (Wahlberg et al 1987). The UW solution, which has found widespread clinical use allows effective preservation of most organs for at least 24 hours and in most cases for 2 to 3 days (Belzer et al, 1990).

***Table 1-1***: Contents of the University of Wisconsin (UW) solution.

| CONTENTS | CONCENTRATION |
|---|---|
| Raffinose | 30 mmol/L |
| K-lactobionate | 100 mmol/L |
| Hydroxyethyl starch | 50 g/L |
| Adenosine | 5 mmol/L |
| Allopurinol | 1 mmolL |
| Glutathione | 3 mmol/L |
| Insulin | 40 U/ml |
| MgSO4 | 5 mmol/L |
| KH2PO4 | 25 mmol/L |
| Penicillin | 200 U/ml |
| Dexamethasone | 16 mg/L |
| Osmolarity | 310-330 |
| pH | 7.4 |

The objective of organ preservation is to preserve the viability of the organ for as long as possible so that, when transplanted, it will regain immediate function. The issues upon which development of preservation fluids are based include; the hypothermia, the physical and biochemical environment of cold storage, and reperfusion injury.

***Hypothermia***

Hypothermia appears to be one of the most important issues in good quality organ preservation. The organ is cooled to 0 to 4°C as rapidly as possible, as soon as the blood supply has been interrupted, and the storage temperature is maintained at 0 to 4°C. Cooling is usually initiated by flushing out the organ through an artery with cold Ringer's solution or cold preservative. When removed, the cooled organs are more tolerant of the absence of a blood supply. After removal, the organs are usually flushed again with preservation solution and packed in a sterile container, placed on ice, and prepared for transfer.

Hypothermia is effective because it slows down metabolism and suppresses the rate of catabolic reactions that occur spontaneously or that are catalyzed by cellular enzymes. Many enzymatic reactions are slowed to about one half their normal rates by a 10°C drop in temperature. A decrease in temperature from normothermia (37°C) to hypothermia (0°C)

would slow down metabolism to less than one tenth its normal rate (Fuller et al 1991).

The tolerance of the liver and other organs to cold ischemia is considerably greater than tolerance to warm ischemia. Much effort has been made in trying to understand the mechanisms of injury to organs during warm ischemia and on reperfusion. If the mechanisms of injury to the liver during warm ischemia are similar to those occurring during cold ischemia, then methods to suppress warm ischaemic injury could be applied in liver preservation and transplantation. However, some studies suggest that the injury caused by warm ischemia is different from that caused by cold ischemia (Holloway et al 1990). Current work suggests that warm ischemia is more damaging to hepatocellular functions than is cold ischemia, and the latter, on the other hand, causes more injury to the vascular system of the liver, including disruption of sinusoidal lining cells (Mckeown et al 1988).

One way in which cold ischemia induces injury to the liver and other organs is through tissue edema and cell swelling. Under conditions of cold ischemia, ATP is lost and the activity of the membrane-bound ion pumps that control cellular volume is decreased. Thus, cells have a tendency to gain water because of the higher colloidal osmotic pressure inside the cell than outside, which is created by the presence of impermeants such as

proteins, phosphorylated compounds, and other substances that cannot permeate the cellular membrane. The prevention of cell swelling on hypothermia appears to be important in obtaining successful organ preservation (Southard et al 1996).

Further metabolic changes which occur as a consequence of hypothermia include acidosis due to lactate accumulation from anaerobic glycolysis and $Na^+/K^+$ ATPase inhibition at (Raison, 1973). Inhibition of the latter results in intracellular sodium accumulation and water influx resulting in cell swelling and death.

***Physical Factors***

Another principle of successful cold storage of organs is the establishment of an optimal physical environment created by the components of the flush-out solution. The physical environment is defined by the osmolality, impermeant and electrolyte composition, hydrogen ion buffer and pH of the preservative, and colloid components of the preservative. These factors are important in maintaining the structural integrity of the tissue and have less to do with maintaining the biochemical integrity of the tissue.

Organ preservation solutions originally designed for the kidney used saccharides (mannitol, glucose, sucrose) as the primary agent to suppress hypothermically induced cell swelling. These agents suppressed kidney

cell swelling but were not particularly effective in suppressing cell swelling in other organs. These solutions also contained other impermeants such as phosphate, sulfate, or citrate that contributed to suppression of cell swelling. Since the development of these solutions, other impermeants have been found to be superior to the ones previously used. These include gluconate and lactobionate, both of which suppress cell swelling in metabolically depressed tissues. Lactobionate appears to be the most effective agent for cold storage of organs, perhaps because of its suppression of hypothermically induced cell swelling. Studies have shown that lactobionate appears to be one of the essential ingredients of the UW solution (Jamieson et al 1988, Sumimoto et al 1990). For simple cold storage, substitution of gluconate for lactobionate was not effective for liver preservation. It is not clear why lactobionate is such an important component for cold storage of organs. The relationship between lactobionate and the suppression of cell swelling as the primary benefit has been questioned (Holloway et al 1989). Lactobionate effectively chelates calcium and may control the calcium content during cold storage and suppress the activation of calcium-dependent processes (phospholipases, proteases, endonucleases) that could damage ischemic organs. Also, lactobionate has been shown to bind iron; the release of intracellular iron could promote oxygen free radical injury during reperfusion of the liver. The chelation of iron by lactobionate may lower the

iron level in the cell and reduce the injury caused on reperfusion by oxygen free radical production (Southard et al 1996).

The physical environment of the organ is also affected by the electrolyte content of the preservation solution. Collins' cold storage solution contains a high content of potassium relative to sodium. It has been shown that storage of tissues in the cold results in a leakage of potassium from the cell in exchange for sodium in the extracellular environment. The high potassium content was used to suppress the leakage of potassium from the kidney during cold storage. This was thought to be beneficial because the cell would not need to expend energy during reperfusion to re-establish a normal intracellular electrolyte content and to pump out the sodium gained during cold storage. Cold storage solutions that contain a high content of potassium and low content of sodium have been termed intracellular-type solution. The UW solution is an intracellular type cold storage solution and contains a high potassium content. However, organs appear generally to be well preserved in solutions with either high sodium or a high potassium content, as long as cell swelling is suppressed by the presence of an approperiate anion (such as lactobionate).

The necessity of colloids in cold storage solution is controversial. Hydroxyethyl starch is included in the UW solution to counteract the hydrostatic pressure and to suppress tissue oedema. Some studies

however, have shown that omission of hydroxyethyl starch does not decrease the efficacy of the UW solution (Jamieson et al 1988).

***Biochemical Factors:***

Metabolism continues during cold storage albeit at a much reduced rate from that at normal temperature. The result is accumulation of end products of metabolism in the tissue, essential metabolites necessary for regeneration of function on reperfusion are degraded, and high energy compounds are lost. This situation is altered only by simple diffusion and equilibration of agents in the cold storage solution within the cells of the organ. Diffusion does occur, as shown by the increased accumulation of lactate reported in the cold storage solution that surrounds the liver during preservation. Thus, it may be possible to add to cold storage solutions, agents that can affect the biochemical environment of the tissue and can improve the function of the organ on reperfusion.

Agents such as oxidizable substrates, precursors of essential metabolites, enzyme inhibitors, membrane stabilizing drugs, and various cofactors could be incorporated into preservatives to improve the quality and longevity of preservation. This principle was, in fact, one that guided the development of the UW solution.

During simple cold storage, all organs rapidly lose ATP, which is catabolized to purines (adenosine, inosine, hypo-xanthine). A loss of ATP of nearly 90% occurs in most organs (Southard et al 1996). On reperfusion, the purine precursors of ATP are flushed out of the cell; thus, the organ suffers from an energy precursor deficit. The lack of substrates for regeneration of ATP immediately after reperfusion could lead to organ failure. In the UW solution, adenosine was added at a relatively high concentration (5 mmol) with the idea that it would diffuse across the plasma membrane during cold storage and provide a precursor for ATP synthesis on reperfusion.

ATP depletion during hypoxia also results in damage of $Ca^{++}$ pumps which leads to an increase in the cytosolic free $Ca^{++}$. Such an increase has been postulated to initiate a cascade of cellular events that culminate in cell death. Specifically, $Ca^{++}$ influx into mitochondria leads to uncoupling of oxidative phosphorylation with further ATP depletion. $Ca^{++}$ also activates proteases which in turn lead to conversion of xanthine dehydrogenase into oxidase (McCord, 1985). Upon reperfusion, and oxygenation, xanthine oxidase acts on hypoxanthine which accumulates during hypoxia from the breakdown of ATP to produce reactive oxygen intermediates (ROI), such as superoxide, and the hydroxyl group (figure-I).

Organs stored anoxically have been shown to lose glutathione in its reduced form, a critical cellular metabolite necessary for numerous reactions and especially in neutralising ROI activities. Some studies suggest that glutathione is critical for successful long-term liver cold storage (Boudjema et al 1990). Omission of reduced glutathione has been shown to lead to failure of rat livers transplanted after 24 hours of cold storage and dog livers transplanted after 48 hours of cold storage.

Further attempts to improve on the current state and achievements with UW solutions have continued. Adding of Lazaroid U74006F ( 21-aminosteroid) a potent inhibitor of iron-dependent peroxidation with antioxidant activity to UW solution in rats reduced releases of transaminases from hepatic graft postoperatively (Cozenza et al 1994). Preliminary studies suggest an advantage of adding the immunosuppressive drug tacrolimus (FK506) to UW solution. The mechanism of its cytoprotective effects is thought to be non-immunological via improving tissue-ATP recovery which would minimise predominantly the warm ischaemia damage (Okano et al 1994). Misoprostil, a prostaglandin $E_1$ ($PGE_1$) analogue demonstrated a protective effect against hepatocellular injury in a rat model of ischaemia-reperfusion. The mechanism of action is thought to be inhibition of endothelial cells-neutrophil adherence and ROI generation (Ping et al 1994).

Flushing solutions are used prior to reperfusion of the graft, principally to clear $K^+$ which is present in high concentrations in UW solution. Carolina rinse solution (Table 1.2) has been shown to improve graft survival following prolonged ischaemia in UW solution. The efficacy of this solution seems to depend largely on the presence of GSH, allopurinol, and adenosine, as selective elimination of these components, particularly adenosine, resulted in reduced graft survival (Toledo-Peryera et al 1994).

***Table- 1.2***: Contents of Carolina rinse solution

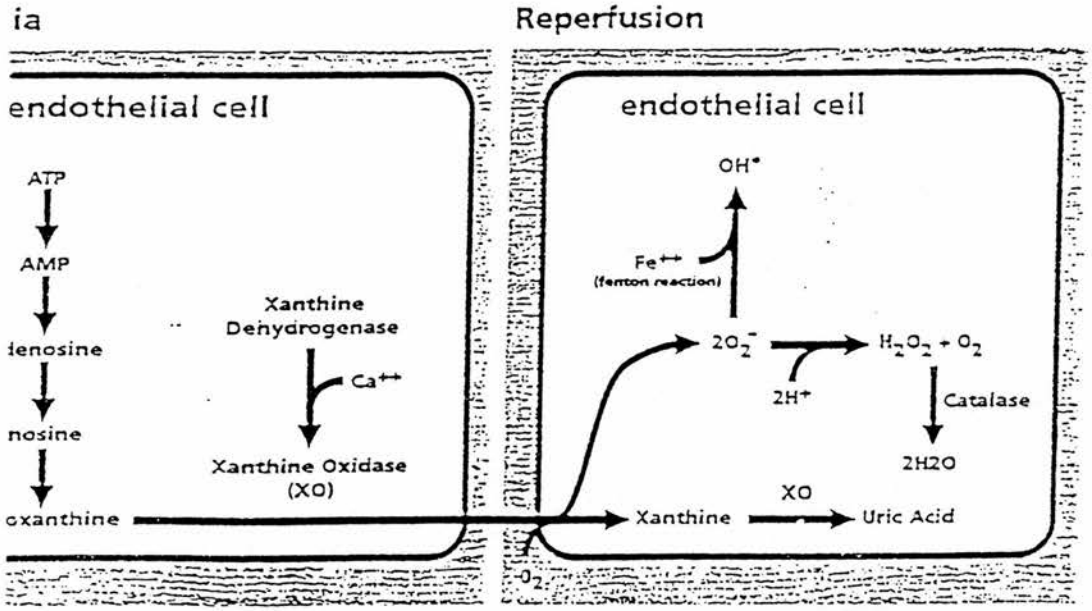
| CONTENTS | CONCENTRATION |
|---|---|
| Hydroxyethyl starch | 50 g/L |
| MgSO4 | 1.2 mmol L |
| KH2PO4 | 1 mmol/L |
| Glutathione | 3 mmol/L |
| Allopurinol | 1 mmol/L |
| Insulin | 100 U/L |
| Adenosine | 0.2 mmol/L |
| Nicardipine | 2 umol/L |
| Desferrioxamine | 1.0 mmol/L |
| Fructose | 10 mmol/L |
| CaCl2 | 1.3 mmol/L |
| KCl | 5 mmol/L |
| MOPS | 20 mmol/L |
| NaCl | 115 mmol/L |
| Glucose | 10 mmol/L |
| Osmolarity | 290-305 |
| pH | 6.5 |

## PATHOGENESIS: REPERFUSION

Tissue damage that follows ischaemia-reperfusion has been the focus of considerable research over the last decade. It has long been recognised that hepatocellular damage after conventional and techniqually successful liver transplantation appears in the early stages of revascularization of the organ. What specifically produces this liver injury has been unclear.

Toledo-Pereyra et al (1975) recognized a well defined clinical and pathological picture present after experimental liver transplantation characterised by post-transplant liver congestion and insufficiency, and it could go as far as thrombosis necrosis and bleeding. In the mid 1970's, the term "reperfusion injury" had not been defined, and the presence of this damage was being referred to by the descriptive physiopathological process such as hepatic outflow block, congestion, insufficiency or thrombosis. It was not until the mid 1980's that the term "reperfusion injury' started to be used more in transplantation (Toledo-Pereyra, 1991).

The no-reflow phenomenon, a descriptive term for reperfusion damage was first used by Majno et al (1967) in relation to the cerebral circulation. Similar changes in the sinusoidal and microcirculatory changes in the liver were later studied in relation to hepatic transplantation injury of the liver. McKeown et al. (1988) and Cadwell-Kenkel et al (1989) were able to




Diagrammatic representation of classical pathway of ROI generation during ischemia/ n. (OH, hydroxyl radical; $H_2O_2$, hydrogen peroxide.)

demonstrate sinusoidal damage through more specific and direct electron microscopy techniques. The damage to the non-parenchymal hepatic cells of rat livers following ischaemia-reperfusion was identified by Thurman et al (1988) using trypan blue dye. Manner et al (1990) demonstrated by direct techniques, that the hepatic tissue perfusion measured by hydrogen clearance, showed a significant difference before and at 30 min and 20 h after transplantation. Livers that had a perfusion rates below 60 ml/100 gm/mm did not have a very good survival and a significant reduction of the microcirculation was noted.

Koo et al (1992) elegantly, demonstrated the same reperfusion changes in the liver sinusoids using in-vivo fluorescence microscopy. They showed that reperfusion of rat livers after a period of ischaemia resulted in adequate microcirculatory blood flow for the first 10 minutes after reperfusion but after 30 minutes complete stasis was seen in the great majority of sinusoids. Ultrastructural examination of the sinusoidal lining cells after iscaemia-reperfusion revealed rounding of cells, focal denudation, inflammatory cell adhesions and platelet aggregation.

In the last decade, considerable attention has been attracted to the role of ROI as mediators of cell injury associated with reperfusion of ischaemic tissue, and it is only recently that the role of other active mediators such as leukotrienes, adhesion molecules endotholins and platelet activating

factors has been explored. In the following section, the relative role of these agents and their inter-relationship in the context of reperfusion injury is discussed.

***Reactive Oxygen Intermediates (ROIs)***

ROIs also known as free radicals are characterised by the presence of one or more unpaired electrons in their outer orbits which make them highly reactive The formation of highly reactive oxygen-containing molecular species is a normal consequence of a variety of essential biochemical reactions. The oxygen free radicals (i.e., superoxide anion. hydroxyl radical, singlet oxygen, and hydrogen peroxide) are capable of extensive tissue and endothelial damage. Superoxide anion is the best studied free radical species ( Pryor, 1976). This free radical may act as a reducing agent donating its electrons or as an oxidizing agent, in which case it is reduced to hydrogen peroxide.

The production of the superoxide radical is a double edged sword. It is beneficial when produced by activated polymorphonuclear leukocytes and other phagocytes as an essential component of their bactericidal activities (Babior, 1978). Nevertheless, if allowed to proceed to excess, the same action may result in tissue damage associated with inflammation (Petrone et al, 1980). Free radicals have been implicated in the development or exacerbation of many human diseases, including ischemia-reperfusion

injury in myocardial infarction, organ transplantation, stroke, cancer, and in various inflammatory-immune injuries and the disorders of aging (Halliwell, 1987).

Upon reperfusion, the enzyme xanthine dehydrogenase is converted to its free radical producing form xanthine oxidase (XO) within endothelial and Kupffer cells (Weizorek et al, 1994). Hypoxanthine, a metabolite of ATP which accumulates during hypoxia reacts with oxygen in the presence of XO generating ROIs such as superoxide and the highly reactive hydroxyl group which are formed via the Haber-Weiss reaction, catalysed by transitional metal ions (Halliwell et al, 1992). Figure 1.1

ROI can mediate injury by a number of mechanisms including: indiscriminate, non-specific interactions with adjacent molecules such as lipids, proteins, and nucleic acids (Halliwell et al, 1992); signal transduction resulting in an increased neutrophil chemotactic activity (Brass et al, 1994); and through interaction with nitric oxide forming the highly toxic species peroxynitrite (Ma et al, 1995).

As a result of the potential for free radicals to damage cells and tissues, an intricate system consisting of both enzymes and small molecular weight molecules with antioxidant capabilities has evolved to protect against the adverse effects of free radical reactions. There exists ultimately a critical

balance between free radical generation and antioxidant defenses. The first line of defence are superoxide dismutases, which catalyze the diffusion-limited dismutation of superoxide to hydrogen peroxide and oxygen. Hydrogen peroxide formed in peroxisomes is metabolized completely by the high content of catalase in these organelles (Oshino et al, 1977). Most important for the metabolism of cytosolic and mitochondrial hydrogen peroxide is the selenium-containing enzyme glutathione-peroxidase. This enzyme reduces hydrogen peroxide to water, requiring reducing equivalent from glutathione (GSH). Another part of this second line of defense is the general strategy of cells to keep any potentially redox-active metal tightly bound to transport and storage proteins to minimize the possibility of hydroxyl radical formation. Thus, iron is transported bound to transferrin or stored as ferric iron bound to ferritin (Minotti et al, 1989). Other defense mechanisms include chain-breaking antioxidants, e.g. vitamin C and $\alpha$-tocopherol (vitamin E), which interrupt the progression of free radical chain reactions by forming stable radicals.

Studies in both humans and animal models have shown ROI generation following reperfusion and anti oxidants such as allopurinol and superoxide dismutase have been shown to significantly reduce reperfusion injury. In a study by Toledo-Pereyra et al (1989) several groups of rats were included to determine the effects of allopurinol, allopurinol and hypoxanthine, XO and allopurinol and catalase. Following pretreatment with all these

different compounds, donor livers were removed, subjected to 20 min of warm ischemia, and allotransplanted into another rat. Indicators of a favourable outcome were seen following transplantation among the rats pretreated with allopurinol, and allopurinol and hypoxanthine, when compared with untreated controls. The findings suggested that inhibition of the XO system, appeared to limit the ischaemic injury in hepatic allograffs following transplantation. Merion et al (1986), studied the effect of allopurinol on tissue XO levels, survival and post-transplant coagulopathy. Donor pretreatment with 100 mg/K/day of allopurinol for 2 days prior to preservation, showed excellent liver protection following transplantation, and XO was reduced by 68% and survival was clearly improved.

***Nitric Oxide (NO)***

This is another important modulator of leukocyte adhesion which has been implicated in the pathogenesis of reperfusion injury. NO is enzymatically synthesized from L-arginine by NO synthase. Its predominant function is relaxation of vascular smooth muscle and inhibition of platelet aggregation (Moncada, 1989). It also inhibits leukocyte-endothelium adhesion by down-regulating the expression of the ligand p-selectin (Gaboury et al 1993), and decreases ROI generation by neutrophils via inhibition of NADPH oxidase. NO is the only known biological molecule that can be produced in high enough concentration to outcompete superoxide dismutase (SOD) for ROI. The interaction results in formation of peroxynitrite ($ONOO^{\cdot}$), a radical in its own right which unlike NO, induces vasoconstriction (Koppenol et al, 1992). It seems possible that NO, via its contribution to generation of $ONOO^{\cdot}$, might indirectly play a role in sinusoidal stagnation and no-reflow phenomenon seen in reperfusion injury.

NO causes vasodilatation via its secondary mediator cyclic guanosine monophosphate (cGMP) which is produced from the activated guanylate cyclase in endothelial cells (Niederberger et al, 1995). ROI have a direct inhibitory effect on guanylate cyclase and result in a reduction in

intracellular cGMP levels (Chevvy et al, 1990), and as such microcriculatory vasoconstriction and stasis.

Pinsky et al (1994) demonstrated elegantly the changes in NO during ischaemia-reperfusion. ECs exposed to a period of hypoxia followed by reoxygenation demonstrate a striking reduction of NO levels and loss of the ability to inhibit thrombin-induced platelet aggregation and $^{14}C$-serotonin release. Addition of the antioxidant superoxide dismutase to the reaction mixture, largely restored the ability of reoxygenated ECs to inhibit thrombin-induced platelet aggregation. These findings further support the hypothesis that NO reacts rapidly with superoxide, generated by reoxygenated endothelial cells or within the vasculature during reperfusion of an organ, leading to depletion NO and hence vasoconstriction, and microcirculatory stasis.

***Endothelins:***

The endothelins are a family of vasoactive peptides with extremely potent and characteristically sustained vasoconstrictor and vasopressor actions in animals and man (Yanagisawa et al, 1988). Three isoforms have been categorized (ET-1, ET-2, and ET-3), and all are of 21 amino acid but differ in two and five residues, respectively. Endothelin-1 was the first of the family to be isolated, and is the predominant isopeptide generated by the vascular endothelium (Luscher et al, 1992).

Exposure of vascular endothelial cells to diverse stimuli, including other vasoactive hormones, cytokines and physical factors, causes increased transcription of the gene for pre-proendothelin-l, a 212 amino acid peptide cleaved intra-cellularly to a 38 amino acid precursor, big endothelin-l. Big endothelin-l is cleaved to endothelin-1 by several isoforms of an unique membrane-bound neutral metalloprotease, endothelin-converting enzyme, which is highly expressed in endothelial cells (Masaki et al, 1991).

Plasma levels of endothelins have been shown to be increased markedly in the reperfusion phase following cardiac ischaemia in rats, as well as in patients undergoing cardiac transplantation (Brunner et al, Lerman et al). Administration of ET-1 to rats resulted in significant hepatic sinusoidal

constriction and a reduction of blood velocity in a dose dependant manner (Okumara et al 1991). ET-1 might therefore have a role to play in the no-reflow phenomenon which has been demonstrated in reperfusion injury.

***Leukotrienes:***

These important inflammatory mediators are metabolites of arachidonic acid produced by the 5-lipoxygenase pathway, primarily in Kupffer cells. The concentration of leukotriene B4 ($LTB_4$ ), a potent chemoattractant increases markedly following ischaemia-reperfusion (Mangino et al 1989, Goldman et al 1990). Oxidants have been reported to activate the plasma membrane phospholipase A2, via an increase in the intra-cellular free calcium (Gyllenhammar et al, 1989), which results in the production of a number of pro-inflammatory agents including leukotriene $B_4$ ($LTB_4$).

There have heen several in vivo studies demonstrating that $LTB_4$ may play an important role in mediating the granulocyte accumulation elicited by reperfusion. Mangino et at. (1989) showed a significant increase in $LTB_4$ production in dog ileal mucosa during reperfusion. In feline intestine, $LTB_4$ production increased two fold following 3 h of isaemia and 1 h of reperfusion (Zimmerman et at. 1990). Additionally, the ischaemia-reperfusion induced increase in neutrophil infiltration (MPO determination) into the mucosa was prevented with either a lipoxygenase inhibitor or an $LTB_4$ receptor antagonist. The leukotriene biosynthesis inhibitor MK-886

has been shown to prevent post ischaemic leukotriene accumulation and the microcirculatory changes of ischaemia-reperfusion injury (Lehr et al, 1991).

***Platelet Activating factor (PAF)***

This is an autacoid phospholipid with vaso-active and pro-inflammatory activity which has been studied in relation to reperfusion injury. Evidence that PAF may be a modulator of leukocyte adhesion in the post-ischemic intestine is based on the observation that, in animals pretreated with PAF receptor antagonists, the ischaemia-reperfusion induced rate of leukocyte adherence and migration is greatly diminished (Kubes et al, 1990). Others have demonstrated that pretreatment with PAF receptor antagonists prevent ischaemia-reperfusion injury in intestines (Filep et al, 1989, Chavez-Cartaya et al 1994). Further, PAF antagonists appear to be as effective as antioxidants SOD and allopurinol in protecting rat stomach against reperfusion induced mucosal damage (Droy-Lefaix et al. 1988).

Although the mechanism of PAF production is unclear, Lewis et al. (1988) reported that cultured endothelial cells exposed to ROIs such as hydrogen peroxide ($H_2O_2$) produce PAF, and thus promote PMN adherence.

***Neutrophils***

An association between neutrophils and cell injury in ischaemic-reperfused tissue is supported by histo-pathological studies showing neutrophil accumulation in the injured region (Engler et al, 1986). Their accumulation has been demonstrated to be reduced by anti-neutrophil serum or monoclonal antibodies (Hernandez et al 1987, Korthius et al 1987). Walden et al (1990) demonstrated an improvement in skeletal muscle contractile function after ischaemia-reperfusion, when the neutrophil population was reduced by vinblastin pre-treatment.

The damaging effect of neutrophils on tissues is due to release of proteolytic enzymes (elastases, proteinases and collagenases) from their granules and generation of ROI via the activated NADPH oxidase a membrane associated enzyme complex. Elastase in particular can degrade almost all components of the extra cellular matrix, attacking intact cells and inactivating various proteins such as immunoglobulins, complement and clotting factors (Weis, 1989).

Neutrophil elastase and ROI interact at number of levels. $\alpha_1$-antitrypsin, a natural inhibitor of elastase is inactivated by ROI through post translation oxidation of methionine at position 358 (Met-358), (Janoff, 1985). Neutrophil elastase on the other hand enhances further generation of ROI from endothelial cells by catalysing the conversion of xanthine

dehydrogenase to xanthine oxidase, an action which can be inhibited by elastase inhibitors, $\alpha_1$-antitrypsin and elastinal (Phan et al, 1992). Such interactions between these mediators potentiates their damaging influence on tissues affected.

The process of neutrophil infiltration originates with an interaction with the endothelial lining cells of sinusoids where the maximum damaging effect is exerted. The nature and magnitude of these adhesive interactions depend upon the expression of various mediators such as the adhesion receptor/ligand molecules, LTB4, PAF, and cytokines such as TNF$\alpha$, $IL_1$, and $IL_2$ (Bagglioni et al 1989, Strieter et al 1989, Geng et al 1990, Lorant et al 1991, Steinhoff et al 1993).

Granule membrane protein (GMP140), and endothelial leukocyte adhesion molecule (ELAM-1) both members of the selectin family increase significantly in the early phases of reperfusion during liver transplantation, to be followed a few hours later by expression of other adhesion molecules such as the vascular cell adhesion molecule (VCAM-1), and the intercellular adhesion molecule (ICAM-1) (Steinhoff et al 1993).

ROI have been shown to promote adherence of leukocytes to the microvascular endothelium and this effect is thought to be mediated via induction of adhesion molecules expression such as GMP140, and also by

activating $PLA_2$ which in turn increases $LTB_4$ synthesis (Lehr et al 1991, Patel et al 1991). The interaction between ROI and adhesion molecules in the process of leukocyte-endothelium adhesion has been demonstrated in a feline intestinal muscle model. Upon superfusion with hypoxanthine - xanthine oxidase a rapid and significant increase in the number of rolling leukocytes, an early and essential event prior to adhesion, was evident. However use of P-selectin monoclonal antibodies prior to superoxide exposure completely inhibited the recruitment of rolling leukocytes (Gaboury et al 1994).

***Kupffer Cells:***

The important role of Kupffer cells in ischaemia reperfusion injury has been demonstrated in a number of studies (Lemaster et al 1995). In a rat model, when hypoxia was selectively induced in pericentral regions of the liver, electron microscopic analysis of Kupffer cells revealed numerous lamellapodia and pseudopodia indication activation. This activation can be prevented by pre-treatment with methyl-palmitate (Lindert et al, 1992).

Following activation, Kupffer cells release a number of inflammatory mediators such as $IL_1$, and $IL_8$ which attract neutrophils, and also produce ROI (Kobayashi et al 1992, Suzuki et al 1994, Hisama et al 1995). Activated Kupffer cells also release TNF$\alpha$ which has been shown to induce production of ET-1 by endothelial cells. Inhibition of Kupffer cell activation

using gadolinium chloride or adenosine-2 receptors agonists such as nisoldipine and prostaglandin $E_1$ has been shown to prevent the microcirculatory disturbances following reperfusion (Arii et al 1994, Reinstein et al 1994)

***Stellate Cells***

The hepatic stellate or Ito cell is located in the space of Disse and resembles the pericytes localised around capillaries. Its role in the regulation of sinusoidal liver blood flow is increasingly recognised. Stellate cells contract in response to a variety of vasoactive substances including Endothelin-1, and substance P (Sakamoto et al 1993, Takemura et al 1995). They are also activated by free radicals (Lee et al 1995). Several vasoactive substances are released during reperfusion injury which may potentially stimulate stellate cells causing sinusoidal contraction with subsequent stasis, hypoxia and further release of inflammatory mediators to complete the vicious circle leading to graft dysfunction.

**Summary:**

There is an increasing evidence from studies on animal models of liver translantation that reperfusion following a period of ischaemia could lead to tissue injury enough to cause graft failure. It also appears that iscahemia/reperfusion is associated with a release of a large number of mediators, which are capable individually or collectively, perhaps in a

cascade fashion, of activating Kupffer cells and initiating neutrophils rolling and adhesion to sinusoidal endothelial cells. The important mediators of chemoattraction, adhesion, transmigration and activation of neutrophils are PAF, $LTB_4$ and the selectin family of the adhesion molecules. The regulation and increased expression of these mediators have been shown both in-vitro and in-vivo studies to be enhanced by ROI. It is plausible therefore, to suggest that ROIs are the main determinant for setting up and triggering the chain reaction which culminates in the activation of neutrophils and Kupffer cells.

**Rationale and introduction to the studies presented in this thesis:**

It appears that ischaemia-reperfusion is a critical stage in organ transplantation generally and liver transplantation is unlikely to be an exception. The humoral and immunological changes during the transplant operation as described earlier suggest that ROI and neutrophils are probably the main determinant of ischaemia-reperfusion leading to graft dysfunction post-transplantation. In chapters II of this thesis, the changes in ROIs during liver transplantation are investigated, and the study assessed their role on graft function and outcome.

Chapter III of this thesis is concentrating on the changes of neutrophil activity using neutrophil elastase as a specific marker. The thesis also assessed the changes in plasma levels of soluble thrombomodulin. This is a cleavage product of a surface protein on the membrane of vascular endothelial cells which is released into the circulation by the action of proteases (e.g. neutrophil elastase), and is considered as a marker of endothelial cell damage.

In chapter IV, the potential role of indocyanine green (ICG) clearance test as an early predictor of graft function is investigated . So far, the available tests and assessment criteria are inadequate in determing the early outcome of transplant operation. A pilot study from our unit (Jalan et al 1994) suggested a possible place for this dynamic test in post-transplant

management. This study is an extention to the pilot one with particular emphasis on the relation of the changes in ICG to ROI and elastase in the context of ischaemia-reperfusion.

Chapter V covers the haemodynamic changes during transplantation and assessing the potential role of two major mediators of vasomotor control; the endothelins, the most potent biological smooth muscle vasconstrictors, and guanosine 3`, 5`-cyclic mono-phosphate (cGMP) the secondary messenger of NO as well as atrial natriutic peptides (ANP) which mediates vasorelaxation. I became interested in doing this study when two of our patients went into profound haemodynamic instabilities during liver transplantation and specifically in the early part of the reperfusion phase. Both patients unfortunately died during the operation. Their haemodynamic changes were typical of the reperfusion syndrome which is described in detail in chapter V of this thesis. Similar pattern and sequence of the haemodynamic alterations post-reperfusion occured in other patients which were transient and of mild to moderate degree. The hypothesis of a role of vasomotor mediators NO (and its secondary mediator cGMP) and endothelins in the pathogenesis of the haemodynamic instabilities in these patients was supported by the studies that showed changes in the activity of the above mediators during experimental liver transplantation.

# CHAPTER TWO

## GRAFT DYSFUNCTION FOLLOWING LIVER TRANSPLANTATION - ROLE OF FREE RADICALS

INTRODUCTION

MATERIALS & METHODS

Markers Of Free Radical Activity

*-The percentage molar ratio (PMR)*

*-Spin Trapping Technique*

STATISTICAL ANALYSIS

RESULTS

DISCUSSION

# INTRODUCTION

In the last decade, a considerable interest has been directed to the role of ROIs as mediators of cell injury associated with reperfusion of ischaemic tissue. The main sources of ROI generation include; as detailed in chapter I the classical xanthine dehydrogenase-oxidase pathway, and the activated inflammatory cells (macrophages and neutrophils) through the surface membrane NADPH.

Koo et al (1992), showed evidence of hepatic microcirculatory stasis which was associated with a significant degree of hepatocellular necrosis following ischaemia-reperfusion in rats. Such effects have been shown to be prevented by concomitant administration of superoxide dismutase (SOD), a free radical scavenger. The evidence of improved graft survival by Carolina rinse and UW solution which contain antioxidants including allopurinol and glutathione further supported the idea that ROIs are involved in the mechanism of graft failure. However, the physical evidence for ROIs generation following human orthotopic liver transplantation and the causative relationship with graft dysfunction has been lacking.

## AIMS:

We aimed in this study to quantitate free radical activity during OLT and to identify the relationship between their magnitude of change and subsequent graft function.

# MATERIALS & METHODS

We studied 21 patients who underwent OLT. The characteristics of the patients are detailed in table 3.1

Matching of donor liver to the recipient was carried out as per standard protocols. Mean donor age was 38 ± 6 (SEM) years. 11 grafts were retrieved by the same team from our unit, while the rest (10 grafts) were retrieved by a team according to the geographical location of the donor. Generally, each team has followed a standard protocol of retrieval. Grafts with significant degree of steatosis or of donors with a history of drug/alcohol abuse were rejected.

University of Wisconsin (UW) solution was used for preservation, and grafts were flushed with Carolina rinse solution prior to restoration of blood flow. All patients underwent veno-venous bypass during the anhepatic phase; mean duration 70 ± 11 minutes.

Following OLT, data collected included daily rate of:

Bile production.

Serum alanine ransaminase.

Prothrombin time.

Cold ischaemia time.

Blood transfusion requirements.

Episodes of acute graft rejection of which treatments were indicated.

For objective assessment of graft function and outcome, we used criteria described previously (Gonzalez et al, 1994), and this is detailed in table: 3-2. The scoring system of this criteria is dependent on serum ALT and prothrombin time activity determined at 72 hours post-operatively, along with the mean value of bile output over the first three days of transplant. In each patient, the score was calculated from the sum of assigned values from each parameter, ranging from 3-9. According to this criteria, Patients with a total score up to 6 were shown to have good outcome and a score of 7-9 is associated with poor graft function. The relationship of these parameters with the degree of free radical activity was determined.

## Markers Of Free Radical Activity:

***The percentage molar ratio (PMR)*** of diene conjugated linoleic acid (PL-9, 11-LA) to its substrate phospholipase-esterified linoleic acid (PL-9, 12-LA) was used as a determinant of free radical activity. Blood samples were taken from each patient through an indwelling catheter inserted following induction of anaesthesia with its tip being positioned in the right atrium. Samples were taken following induction of anaesthesia, during anhepatic phase and following reperfusion at 30 minutes, 1, 2, 6, 9, 12 and 24 hours. Plasma was separated by centrifugation at 3000G for 15 minutes and stored at -40°C for later analysis. Plasma concentrations of both (PL-9, 11-LA) and (PL-9, 12-LA) were measured by high performance liquid chromatography after enzymatic hydrolysis, and solid phase separation as described by Iverson et al (1985):

## *Material:*

Tris and phospholipase $A_2$ (EC 3.1.1.4) from *Naja naja* venom were obtained from Sigma, Poole. Dorset. UK. Methanol. acetic acid. acetonitrile (grade 'S'). propan-2-ol and water were obtained from Rathburn Chemicals. Peebleshire, UK. Isolute octadecyl disposable sample preparation columns were obtained from Jones Chromatography. Llanbradach. Mid Glamorgan. UK. The methyl ester of 18:2(9.I1) prepared from dehydrated castor oil was obtained from the Paint Research

Association, Teddington Middlesex, UK. HPLC was performed on a Watters 510 pump, Perkin Elmer Dioor array ultraviolet (UV) detector set at wavelengths 234 and 205 nm. 50 μL samples were injected into columns (A Spherisorb ODS2 250 X 4 mm column containing 5 μm capped spherical particles) using a Perkin Elmer IS100 autosamplers). A mobile phase of acetonitrile/water/acetic acid *85:15:0.1* at a flow rate of 1.5 mL/min was used. Chromatograms were integrated and quantified using a Kortran Integration Pack.

## *Method:*

0-5 mL plasma was mixed with 0.5 mL of a solution comprised of 0.1 moL Tris, pH 8.9 mol/L methanol and 5000 U/L phospholipase $A_2$, which was stable at $4^0$C for 3 weeks. The mixture was incubated at $25^0$C for 15 min after which 2 mL methanol containing 0.5% acetic acid and 50 mg/L beta-eleostearic acid [I8:3(9,I1,13)] was added to precipitate the protein and add internal standard. This solution was stable at $-20^0$C in a dark bottle for 3 months. The preparation was then centrifuged and 2 mL supernatant applied to a 'Bond Elut column that had been washed twice with 2-5 mL propan-2-ol/acetonitrile 2:1 and conditioned twice with 2.5 mL of a wash solution of methanol/water/acetic acid 67:33:0.04 immediately before use. The 'Bond Elut' column was washed again and the sample was eluted with 1 ml propan-2- ol/acetonitrile 2:1. The eluate was injected directly into the

HPLC filling the sample loop. The conjugated dienes were measured at 234 nm and the nonconjugated fatty acids (linoleic, linolenic and arachidonic acids) were measured at 205 nm. The coefficient of variation of the assays was less than 3.5% and results are expressed as diene conjugated /linoleic acid ratio.

## *Spin Trapping Technique:*

In a subgroup of 10 patients, we used a spin trapping method as another determinant of free radical activity. This has been described previously (Grech et al, 1993). In brief, from each patient, blood samples (one ml each) were withdrawn from the same right atrial catheter after induction of anaesthesia, during the anhepatic phase and following reperfusion at 1, 12 and 24 hours. They were immediately mixed with 50mM solution of the spin trapping agent $\alpha$- phenyl N-tert-butyl nitrone (PBN) and stored in liquid nitrogen. The PBN-free radical adduct was extracted by adding 2 ml of toluene to each ml of the mixture followed by centrifugation at 10,000G for 5 minutes. The toluene extract was separated and stored at -40°C until analysis by electron paramagnetic resonance (EPR) spectroscopy. EPR was measured by Bruker ECS106 spectrometer. The microwave power was 20mW, applied field, 3355 $\pm$ 30 gauss and frequency of 9.4G Hz, using 100KHz modulation with an amplitude of 0.5 gauss. Time constant was 0.3s and sweep time of 84s. Sample temperature was 220°K. Free

radical concentrations were determined by comparing the double integrals of the EPR signals from the experimental samples with those from known concentrations of a stable free radical, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, in the same solvent and recorded under identical conditions. In those cases where very low signal:noise precluded accurate integration, radical concentrations were estimated by comparison of signal heights of the weaker signals with those of the stronger signals.

## STATISTICAL ANALYSIS:

Results were expressed as mean $\pm$ standard error (SEM). Statistical significance was accepted for $p<0.05$. Difference between time points was calculated using one way analysis of variance. Correlation between variables was performed by linear regression analysis using the Spearman correlation coefficient.

## *TABLE 2.1*: PATIENTS CHARACTERISTICS

| | |
|---|---|
| Number | 21 |
| Mean Age | 53 ± 3.8 years (SEM) |
| Sex (M:F) | 10 : 11 |
| Indication for transplantation | |
| Primary biliary cirrhosis | 9 |
| Primary sclerosing cholangitits | 3 |
| cryptogenic cirrhosis | 3 |
| Alcoholic liver disease | 6 |

***Table 2.2*** Parameters of scoring system for assessment of early postoperative graft function in liver transplant patients.

| Parameter | Assigned value |
|---|---|
| Serum ALT (U/L)[a] | |
| < 1,000 | 1 |
| 1,000-2,500 | 2 |
| >2,500 | 3 |
| Bile output (ml/day)[b] | |
| > 100 | 1 |
| 40-100 | 2 |
| < 40 | 3 |
| Prothrombin activity (%)[c] | |
| > 60 (spontaneous) | 1 |
| > 60 (with fresh-frozen plasma) | 2 |
| < 60 (despite fresh-frozen plasma) | 3 |

Footnote: [a]Highest value in the 72 hr after transplant. Normal value is up to 40 U/L. [b]Mean value during the first 72 hr after transplant.
[c]Lowest value in the 72 hr after transplant.

# RESULTS

Figure 2-I, shows the intra-operative changes in free radical activity as measured by diene conjugated method. The mean base line plasma PMR was 10.87 $\pm$ 0.78 % which although higher than those of healthy volunteers (9.74 $\pm$ 0.84 %), did not reach statistical significance. No significant change in activity occured during anhepatic phase (10.29 $\pm$ 0.71 %) but a marked rise in activity was evident following reperfusion with an initial peak at 1 hour (24.42 $\pm$ 7.81 %, $p< 0.01$). A second more prominent peak appeared 12 hours post-reperfusion following a transient decrease of radical activity (21.91 $\pm$ 2.46%, $p< 0.01$). Figure 2-2, represents the HPLC measurements of a patient before and after transplantation.

Using the spin trapping technique, PBN-adduct signal were barely detectable after induction of anaesthesia and during anhepatic phase (figure2-3). A significant increase in intensity was evident following reperfusion with larger signals obtained in 12 hours samples which showed 3 lines from a nitrogen atom, with $a_H$ = 14 gauss and these are split into doublets by a hydrogen atom, with $a_N$ = 2 gauss. Such signals are identical to those reported by Tortolani et al (1993). Levels of PBN-radical adducts of those samples were 2-3 times higher than the pre-transplant ones ($p< 0.05$). Representative EPR spectra obtained from a patient before and after reperfusion of the graft are shown in figure 2-4.

The PMR ratio representing free radical activity as measures using the diene conjugated method, correlated with alanine ALT at 24 hrs post-transaplantation (figure 2-5), and time to prothrombin time normalization (figure 2-6).

Six patients had poor graft function according to the criteria defined earlier (table 2-I) with scores ranging between 7-9. Of these, three had died on days 7, 12 and 31 days following acute post-transplant liver failure. Another patient from the same group developed features of PNF and required retransplantation on day 3. Figure 2-7, shows the mean delta PMR activity which is the difference between the maximum and base-line values plotted against total score of graft outcome for each patient. A significant correlation was found, the higher free radical activity the worse the graft outcome ($p< 0.01$).

There was no correlation between the magnitude of free radical changes and cold iscaemia time (10.61 $\pm$ 0.58 hrs) (fig 2-8) , mean blood units (7.27 $\pm$ 1.12 units) or blood products transfused (26 $\pm$ 5.4 units) (fig 2-9). Acute rejection which required treatment as judged by clinical and histological criteria occured in 7 patients. Mean PMR of patients with rejection was 11.69 $\pm$ 0.84 % compared with 11.17 $\pm$ 1.54 % in the non rejection group (NS) (fig 2-10).

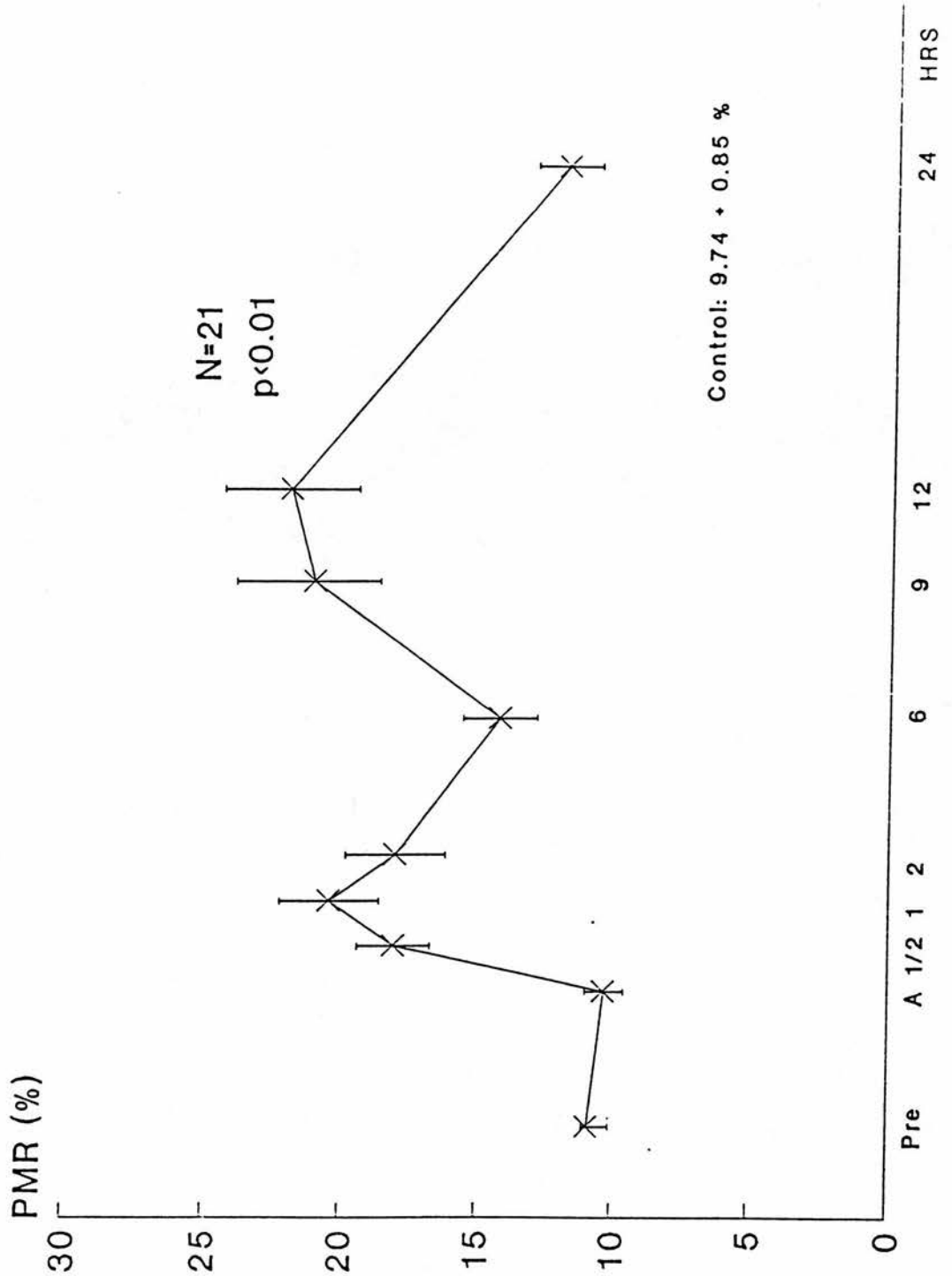



**Figure 2-1**: Perioperative PMR ratio as a determinant of free radical activity. Pre: pre-transplant, A: during anhepatic phase, and then post-reperfusion up to 24 hrs

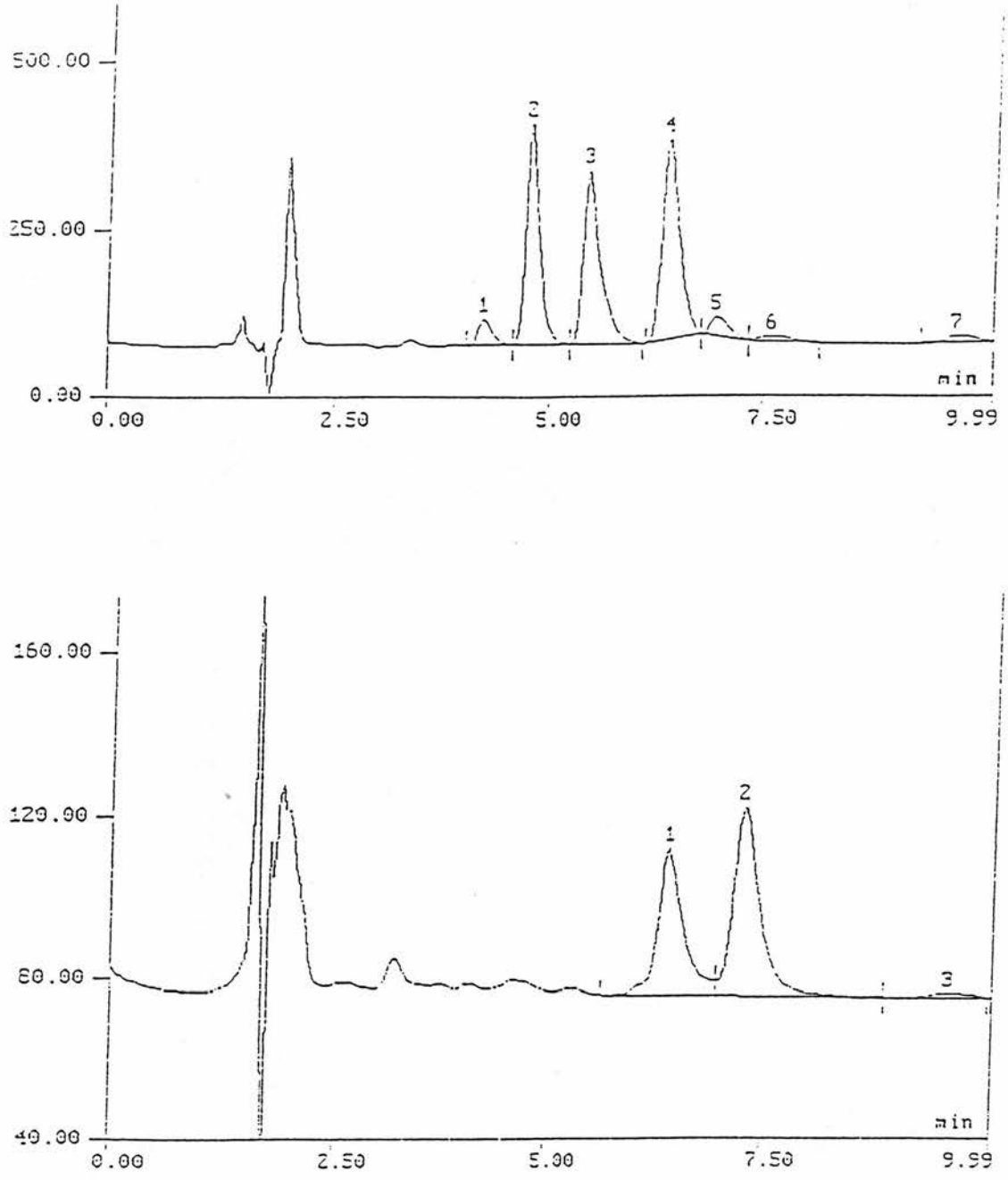



Figure 2-2: These diagrams represent the HPLC measurement of plasma for one of the patients. In diagram (A), peak 4 represents the linoleic acid concentration in the sample, peak 3 is that of arachidonic acid, and 2 for a mixture of fatty acids. In diagram (B), the peak labeled 1 represents the internal standard, and 2 represents the concentration of diene - conjugated derivative

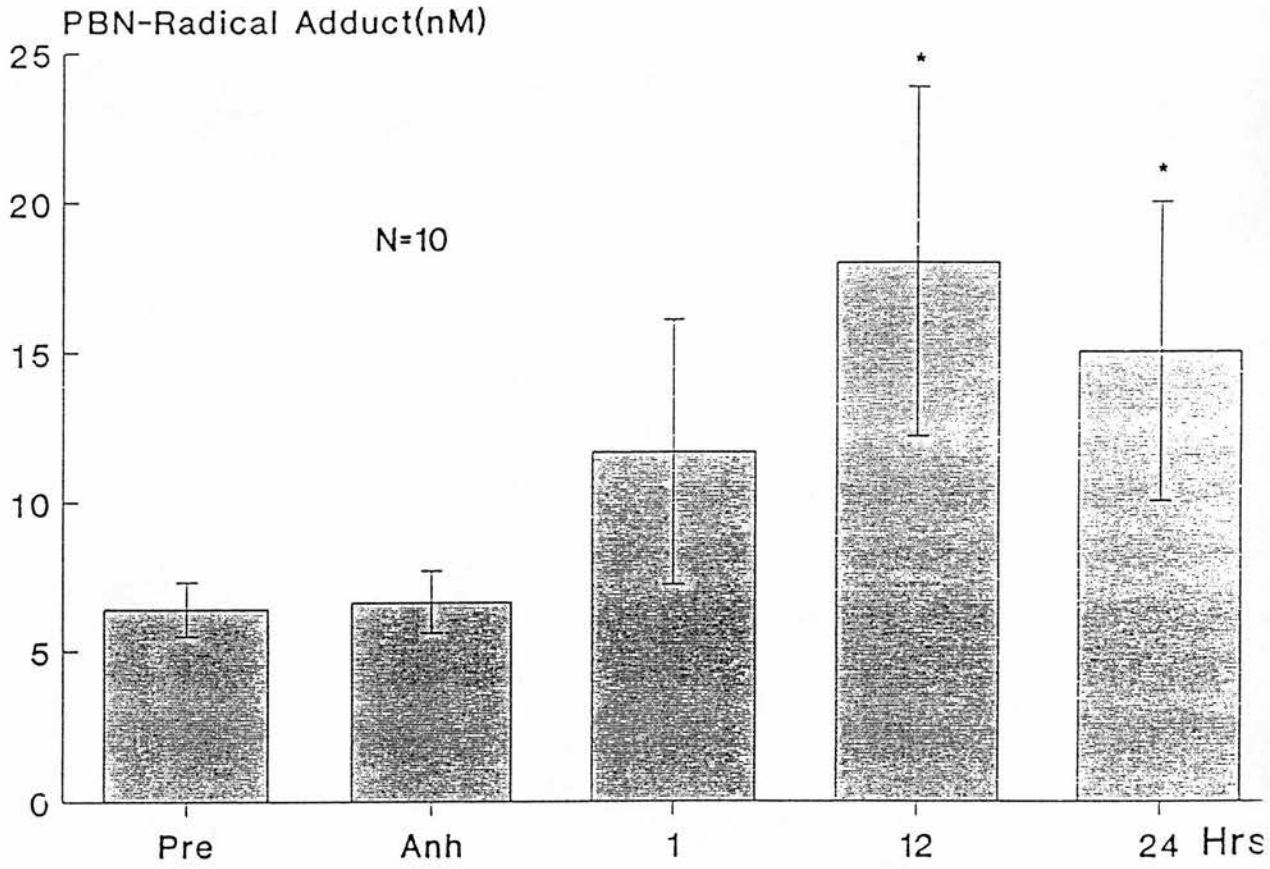



**Figure 2-3**: Peri-operative changes in ROI activity determined by EPR method, and identified as PBN-radical adduct signals. Pre: pre-transplant, Anh: anhepatic phase, and then post-reperfusion at 1,12, & 24 hrs

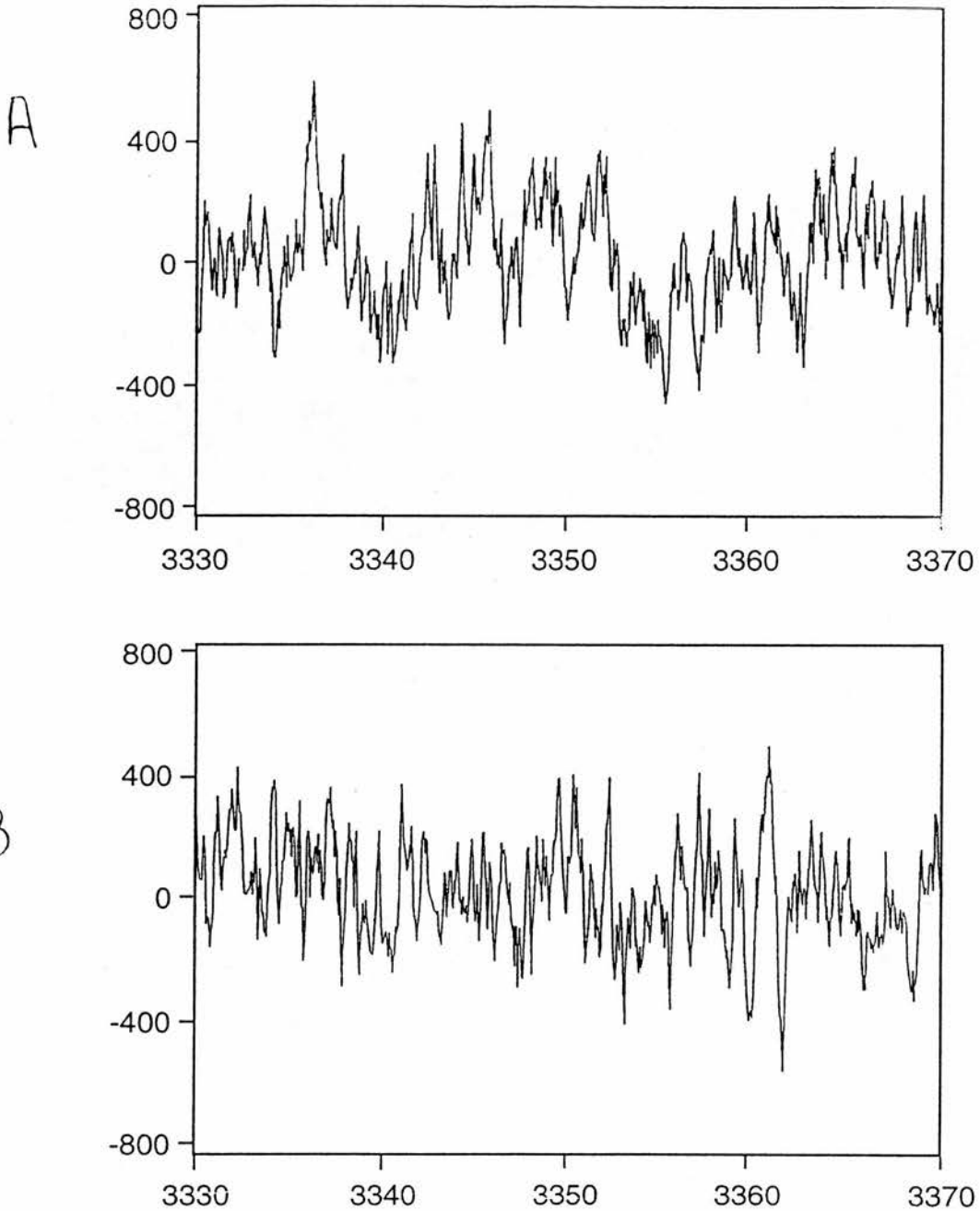
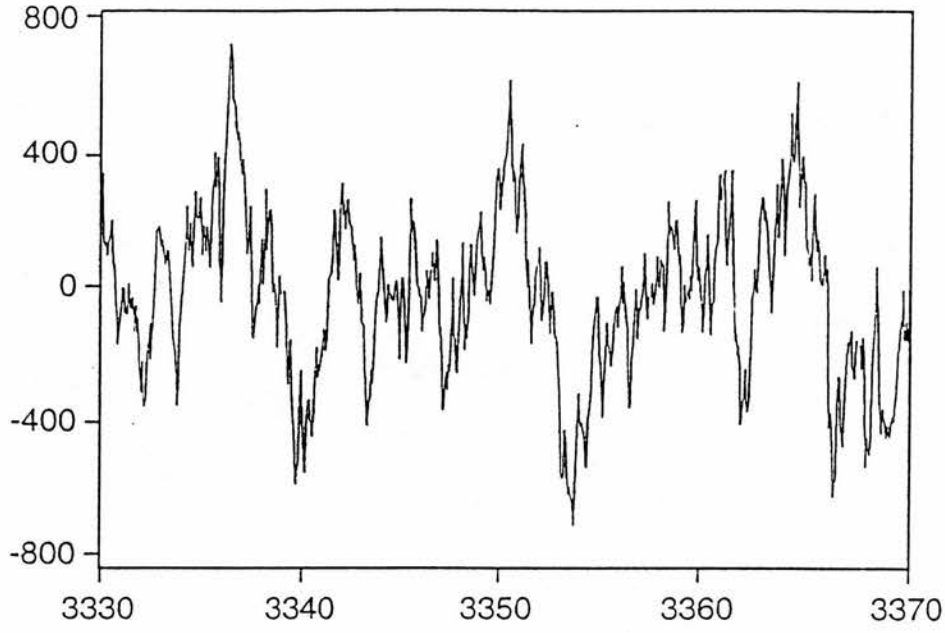



**Figure 2-4:** Representative time course of EPR spectra of PBN-radical adduct from one patient. C & D are shown in the ~~next~~ following page: (A) prior to induction of anaesthesia, (B) during anhepatic phase, (C) 12 h after reperfusion and (D) spectral simulation of the PBN radical adduct, using the parameters $a_H$= 0.19 mT, $a_N$= 1.36 mT. Comparison of this simulation with the spectrum in (C), clearly shows the similarity of the radical species. Spectra (A) and (B) show evidence of the same radical, although at the lower concentration, the hydrogen hyperfine is not resolved

C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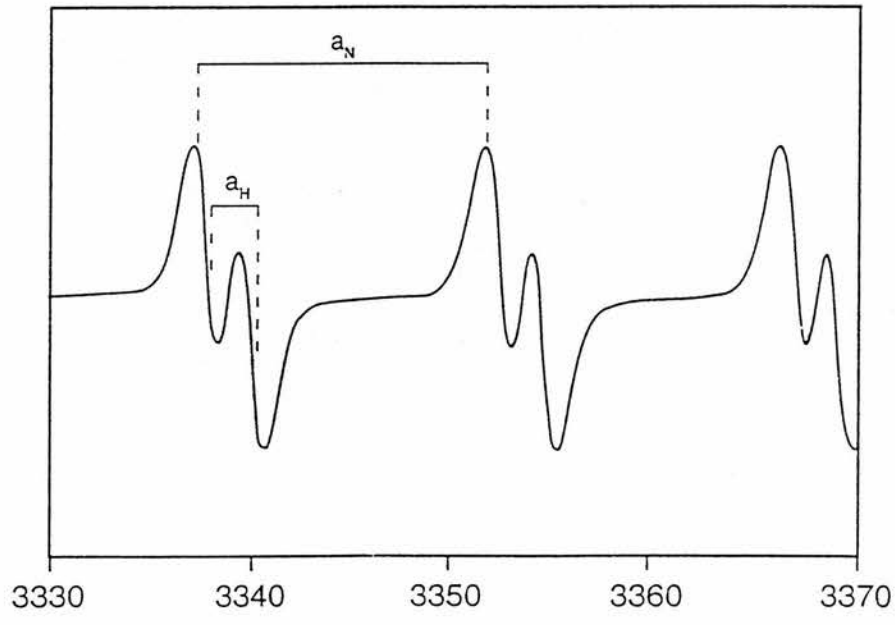
D


$a_N$
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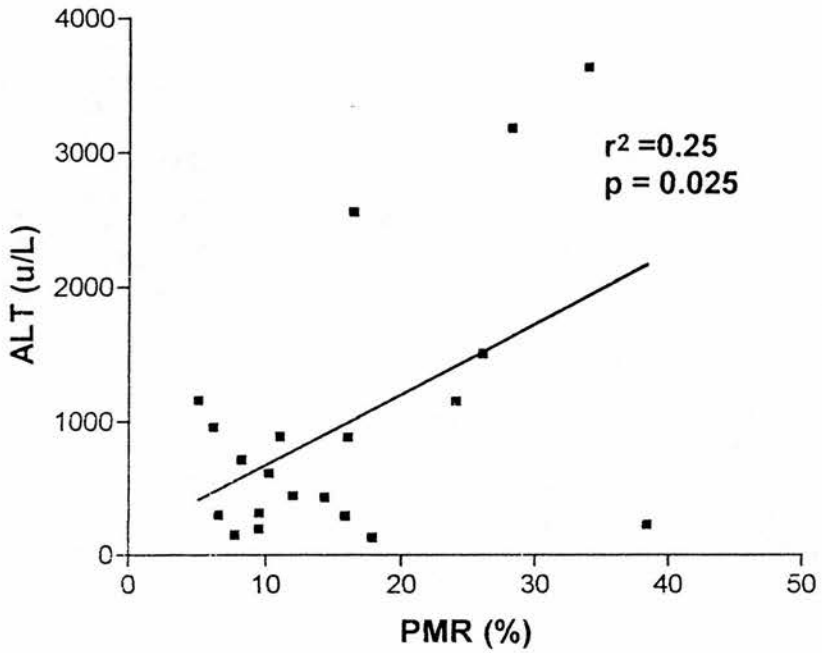



Figure 2-5: The correlation of free radical activity measured by the diene conjugated method and alanine transamainase measurement (ALT) 24 hrs post transplantation

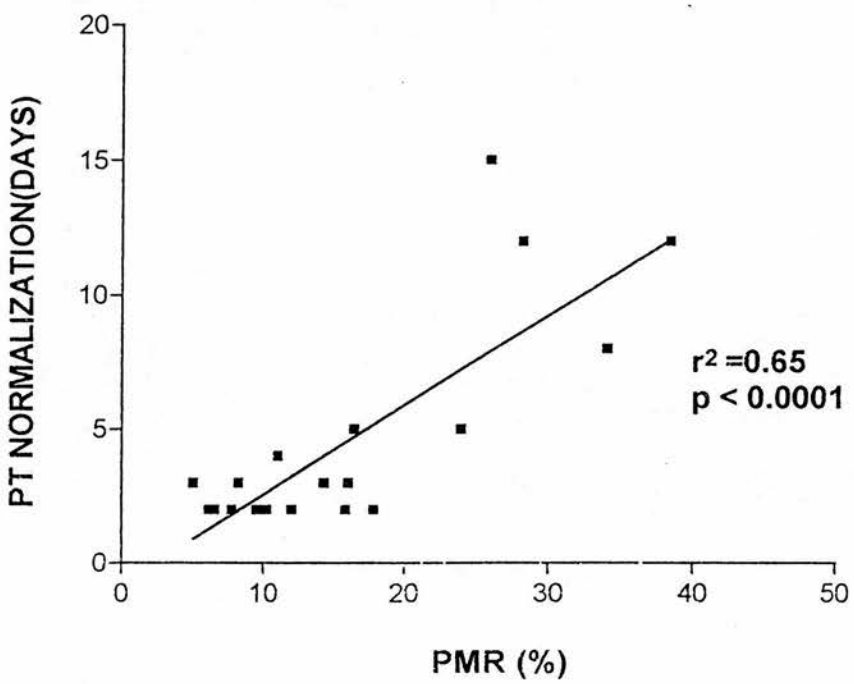
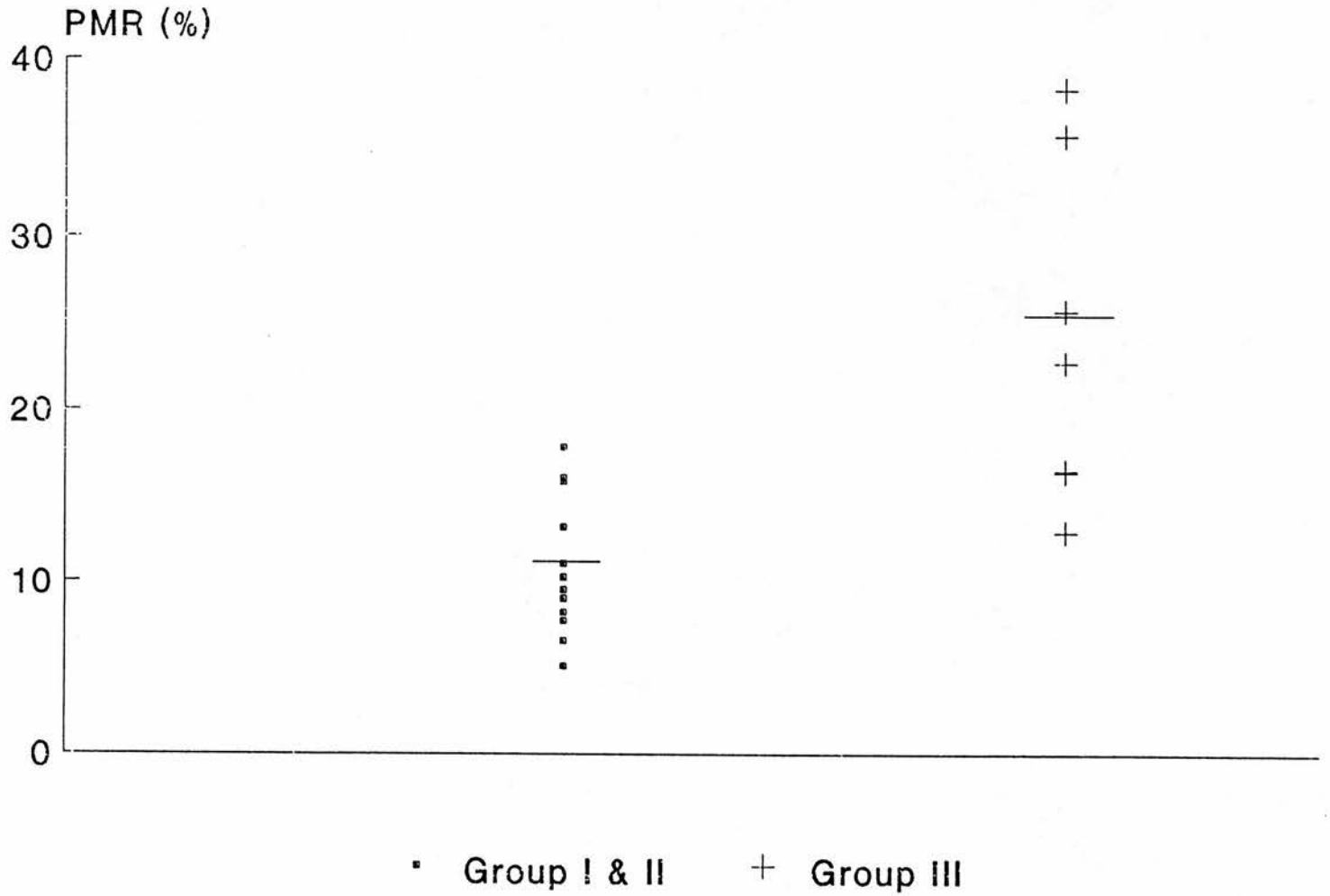
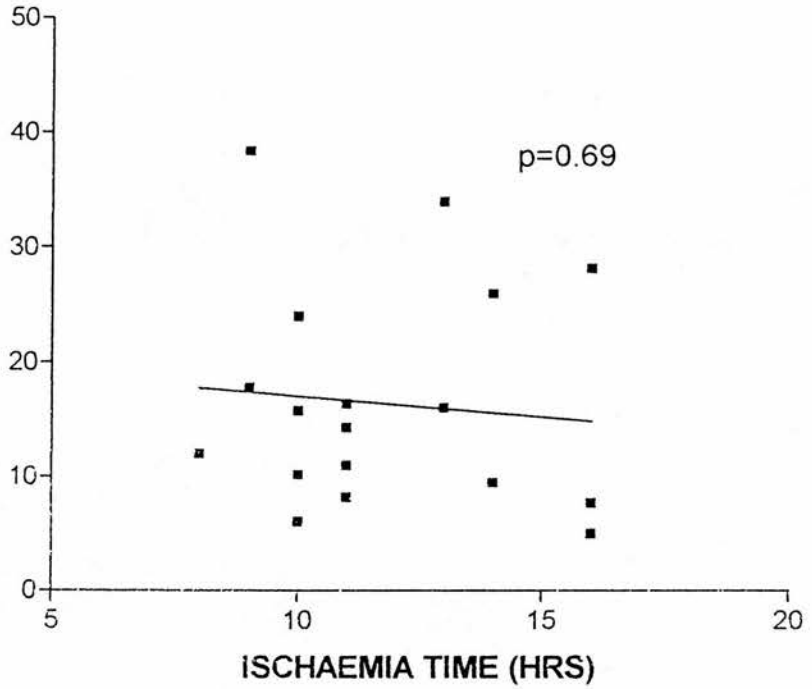



Figure 2-6: The correlation of free radical activity determined by the diene conjugated method and the time to normalization of prothrombin time.

# OUTCOME






e 2-8: This graph demonstrates the lack of correlation
een the cold ischaemia time and the perioperative changes
e radicals

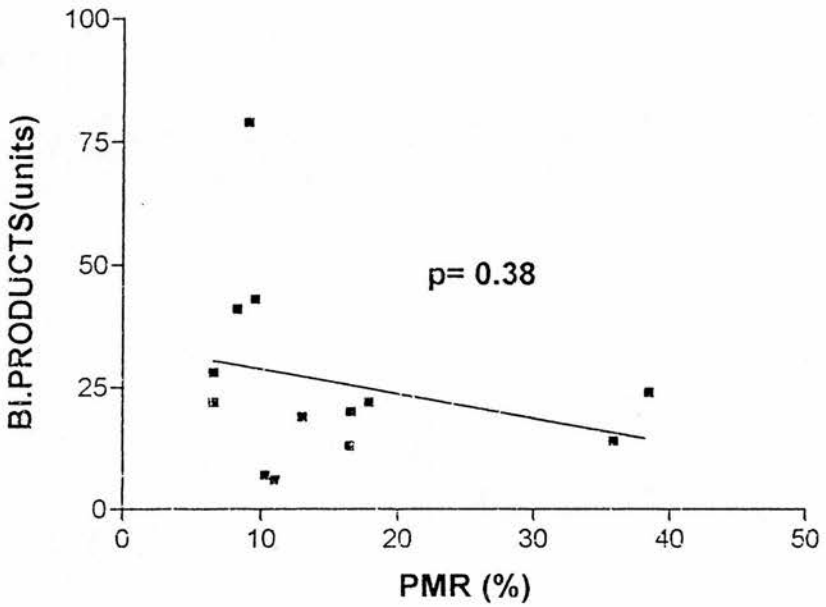



Figure 2-9: Correlation of free radicals activities represented by the percentage molar ratio and the total blood and blood products administred perioperatively

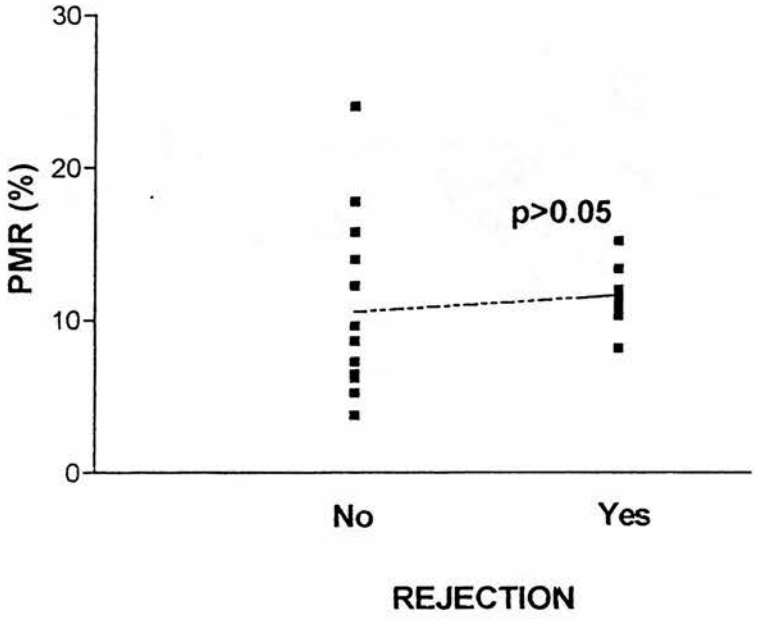



Figure 2-10:This graph illustrates the relationship of rejection episodes and free radical activity determined by the percentage molar ratio(PMR) using diene conjugated method.

## DISCUSSION:

There is increasing evidence to suggest that oxidative stress, the result of excessive production of oxygen-derived and other radicals, is a major cause of reperfusion injury. These observations are based on experimental and animal model studies (Conor et al 1992, Kobayashi et al 1991), and This is supported by favorable effects of antioxidants such as allopurinol, ascorbic acid and superoxide dismutase when used in such situations (Kobayashi et al 1991, Zhong et al 1989).

In this study we have shown evidence of increased free radical activity following reperfusion of the new liver graft. We used two methods to quantitate free radical activity because of the limitations of the individual methods alone as suggested previously (Halliwell et al 1992). The PMR method is regarded as a "fingerprint" assay providing indirect evidence of radical production by determining the end products of lipid peroxidation which in this case is diene conjugated isomer. These methods have been the most frequently used for detecting free radical activity in human diseases for their safety as they are primarily in-vitro techniques. EPR spectroscopy, on the other hand, is a direct and quantitative method which measures the energy changes that occur as unpaired electrons align in response to an external magnetic field (Halliwell et al 1992). As many free radicals have a very short life (less than milliseconds), such measurement

therefore depends first on trapping the radical by administering a compound such as PBN (preferably intravenously) to produce a nitroxide (a spin-trapped adduct) whose life time is considerably longer than that of the parent free radical and therefore detectable by EPR spectroscopy (Holley et al 1993). In human studies toxicity of PBN has limited the technique to mixing of blood samples with the spin trap as soon as possible after collection. Despite the inevitable reduction in the sensitivity of the technique due to the time lag between free radical generation and trapping, it remains by far the most specific in detecting radicals, when compared with the other currently available techniques. Valuable data has been obtained, for example, relating to free radical production during angioplasty (Coghlan et al 1991, Grech et al 1993).

The results of this study have shown significant degree of correlation between the degree of free radical activity and the markers of graft function which is suggestive of a role for free radicals in the aetiology of PGD. The criteria we used in this study (Gonzalez et al 1994) is a modification to the one defined by Greig et al in which the widely accepted parameters for assessement of early post-operative graft function are used. This include serum levels of aminotransferases, bile production and coagulation status, alone or in combination. In this criteria, a well defined cut off values for each parameter is dteremined and it has also included

the use of fresh frozen plasma which often happen in the early post-transplant period as another determinant for scoring.

The currently available knowledge, however does not support the suggestion that reperfusion injury is simply a matter of direct damage to tissues by toxic radicals (Bulkley, 1994). Inflammatory mediators such as leukotrienes, PAF, and soluble adhesion molecules (e.g. p-selectin) have been shown to be upregulated by free radicals (Lewis et al 1988, Patel et al 1991, Reilly et al 1991), and they have been implicated as well in reperfusion injury (Kubes et al 1990, Lehr et al 1991, Steinhoff et al 1993). These molecules react with the complementary ligands on the circulating neutrophils, leading to their arrest and consecutively activation with release of proteases and free radicals (Gyllenhammer et al 1989, Lorant et al 1991). The biphasic appearance of free radical generation seen in this study (figure-I) may support the view of their importance in the initiation of the inflammatory cascade and consecutively reperfusion injury, as the earlier rise of free radicals is likely to result from xanthine oxidase on the endothelial cell surface, via the classic free-radical- mediated chain reaction with the late surge of radicals being a direct product of activated neutrophils.

We did not see a correlation between cold iscaemia time and the degree of free radical activity. Prolonged ischaemia can lead to increased

accumulation of XO and hypoxanthine which subsequently could determine the extent of free radical activity. It is possible that the limited ischaemia time in this study has been a factor and this could be supported by findings of a previous study that showed extended preservation beyond 16 hours in UW solutions is unfavourable and likely to influence subsequent graft function.

Acute graft rejection which required treatment occured in 30% of the patients studied. We did not see a difference in free radical activity in the patients with rejection as compared with non-rejection group. Poor graft function has been shown to increase the incidence of graft rejection (Howard et al 1992). The mechanism for such relationship is however unclear, and from the results of this study it would seem unlikely to be free radical related.

In conclusion, this study provides evidence of increasing free radical activity following reperfusion of liver grafts and the magnitude of changes correlated with the severity of graft dysfunction.

# CHAPTER THREE

## NEUTROPHIL ELASTASE: A DETERMINANT OF ENDOTHELIAL DAMAGE AND REPERFUSION INJURY FOLLOWING LIVER TRANSPLANTATION ?

# INTRODUCTION

The polymorphonuclear neutrophils (PMNs) provide a host defense against bacterial, fungal and other injurious agents that is vital to the survival of the organism. However, if PMN functions become excessive, it can lead to tissue injury. This is illustrated by the increasing body of evidence that implicates these cells as mediators of tissue injury in a variety of inflammatory diseases, including rheumatoid arthritis, ischaemia/reperfusion injury, ulcerative colitis and various skin diseases (Malech et al 1987).

Although early restitution of blood flow to an ischemic tissue is essential to prevent further hypoxic cellular injury, a paradox exists in that it is at the time of reperfusion that much of the injury occurs. Parks et al (1986) demonstrated that the mucosal injury produced by 3 h of ischaemia and 1 hour of reperfusion is significantly greater than that caused by 4 hours of ischaemia alone. Crissinger et al (1989) showed greater mucosal dysfunction in the piglet intestine during the reperfusion period than the ischaemic period. It is now believed that a potential explanation for this observation is that neutrophil infiltration and migration out of the vasculature occurs primarily at the time of reperfusion.

Activated neutrophils cause tissue damage by release of free radicals and proteolytic enzymes such as metalloproteinases and elastase, from the granules. They are capable of direct tissue damage and enzymatic inactivation unless neutralized by their natural inhibitors (Weiss, 1989). Neutrophil elastase (NE) is the most potent and because of its specificity, has been established as a means of assessing in-vivo neutrophil activation (Plow, 1982).

## Endothelial Cell Damage and Thrombomodulin release:

Several in-vitro and in-vivo studies have suggested that the endothelial cell damage by NE results in cleavage and inactivation of surface thrombomodulin (TM) via a specific hydrogen peroxide ($H_2O_2$) facilitating mechanism involving oxidation of Met 388 in the TM molecule (Hiroki et al 1994). This results in the loss of transmembrane and cytoplasmic domains and release of the soluble form into the circulation(sTM).

TM is a surface protein of vascular endothelial cells. When thrombin is bound to TM, its procoagulant properties are reduced and its anticoagulant ability to activate the zymogen protein C increases. Protein C catalyzes in conjunction with its cofactor protein S the proteolysis of the activated coagulation factors Va and VIIIa and inhibits the activity of the

plasminogen activator inhibitor thereby augmenting indirectly fibrinolytic activity (Dittmann et al 1990, Himmelreich et al 1994). Thus, TM plays an important role as an anticoagulant protein on the blood vessel wall. Immunohistochemically, TM has been found to be mainly present on endothelial cell surfaces of blood and lymphatic vessels in all organs except the brain (Maruyama et al 1985, Ishii et al 1986).

A smaller form of TM, the soluble thrombomodulin (sTM), has been isolated from human blood and urine (Ishii et al 1985). The structure of sTM is not known but is thought to be similar to the soluble protein obtained after proteolytic modification of TM with elastase (Kurosawa et al, 1987), a cleaved form of tissue TM with loss of part of the transmembrane domain, and the cytoplasmatic tail (Dittmann et al 1987). Therefore, sTM in plasma appears to be derived from injured endothelial cells or to be proteolytically cleaved from TM by proteases (Himmelreich et al 1994).

## AIMS:

In this study we assessed the degree of proteolytic activity of neutrophils by determining the changes in NE before and after reperfusion in liver transplantation. We looked at the relationship of the changes with the parameters of subsequent graft function. Perioperative changes in sTM was used as a marker of endothelial cell damage.

# MATERIALS & METHODS

We studied 19 patients who underwent OLT. The details, including the indications are summarized in table 3-1.

## Transplant details

Donor age was 42 ± 6 years. ABO blood group compatibility and matching of donor-recipient sizes were observed. As per protocol grafts with macroscopic steatosis or from donors with known history of drug/alcohol abuse were rejected. University of Wisconsin solution was used for preservation.

All patients underwent veno-venous bypass (VVB) during the anhepatic phase. It was started after dissecting the porta hepatis and clamping of the portal vein. The mean duration of VVB was 72 ± 25 min, which was discontinued just before unclamping of the portal vein and reperfusion of the donor liver.

Details of blood and blood products transfusion perioperatively were recorded. Following OLT, data collected included

bile flow, serum transaminases (ALT), and prothrombin time (PT). These were recorded daily up to the time of discharge, retransplantation, or death

Postoperative graft function was assessed using the above parameters and were graded according to the criteria that was described earlier in the method section of chapter III, and summarized in table 3-2. patients with a total score of up to 6 (group I:up to 3, and group II: 4-6) were shown to have good outcome and a score of 7-9 (group III) is associated with poor outcome.

Blood samples were collected into EDTA containg tube, and were taken from an indwelling catheter in the right atrium before transplantation, during the anhepatic phase, and then post reperfusion at 0.5, 1, 2, 6, 9, 12, and 24hrs. Plasma was immediately separated by centrifugation (3000 rpm for 20 minutes) and stored at -40°C for later analysis.

## Neutrophil Elastase Measurement:

***Principle:***

Plasma NE was measured by a specific radioimmunoassay using rabbit polyclonal antiserum (Plow, 1982). The antigen was purified from human neutrophils following leukophoresis. The antibody was absolutely specific for neutrophil elastase with no cross-reaction with pancreatic or platelet

elastase. Neutrophil elastase was measured either as the free enzyme or as a complex with its natural inhibitors, $\alpha_1$-antitrypsin and $\alpha_2$-macroglobulin.

***Materials:***

1. Assay buffer:- This contains; 0.05M phosphate [pH 7.4], 0.6M NaCL, 2 mM EDTA, 20 U/ml (130 μg/ml) heparin, 20μ/ml aprotinin and 2% heat inactivated horse serum.
2. Standard:- Calbiochem purified elastase in normal human plasma, prepared in a solution to give aconcentration of 200 ng/ml.
3. 1st Antibody made to a dilution 1/1500 using the assay buffer.
4. Tracer: The same standard elastase is iodinated in-house using Chloramine T method to give $^{125}$I-elastase at a concentration of 10ng/ml.
5. 2nd Antibody: Donkey anti-rabbit antibodycoupled to a solid phase support (DAR-SP) diluted by the assay buffer to 1/6 concentration.

## *Assay Procedures:*

50μl of the standards or samples were added to the tubes (labelled previously) containing 50μl $^{125}$I-elastase (10ng/ml), and 50μl anti-elastase (1/1500). The mixture in each tube was made up to a final volume of 200

μl by adding 50μl of the buffer solution. Samples were incubated at room temperature overnight.

In the second day, 50μl of DAR-SP (1/6 dilution) was added to the tubes followed by agitation for 45 min (setting 8).

Each tube, was then washed once with 1ml of the assay buffer, and followed by addition of 1ml sucrose (10%) and left to stand for 20 minutes to allow for sedimentation of the bound complex in the solid phase. The supernatant was aspirated and discarded. The bound complex was then counted using a NE1600 gamma counter. Plasma PMN elastase was expressed in ng/ml and the sensitivity of the assay was 5 ng/ml with a 4% interassay coefficient of variation.

Results from the transplant patients were compared with those from healthy volunteers matched for age, as established in our laboratory reference ranges.

## Soluble Thrombomodulin measurement (sTM)

Plasma antigen levels of sTM were determined using an enzyme immunoassay which was described previously (Amiral 1994).

***Principle:***

A solid support (micro-Elisa plate) is coated with a specific mouse monoclonal antibody which binds thrombomodulin in the assay standard or in the patient's sample. The bound Thrombomodulin is next revealed by the

use of a second mouse anti-thrombomodulin monoolonal antibody labelled with horseradirh peroxidase which binds to another antigenic determinant of the thrombomodulin molecule that is at a distance away from the first one

The bound enzymatic activity is then demonstrated by its oxidative action on the substrate ortho-phenylenediamine (OPD) in the presence of hydrogen peroxide. After the reaction has been stopped by the addition of sulphuric acid, the colouration that is obtained is measured at 492 nm. The observed optical density is directly proportional to the concentration of thrombomodulin.

## *Materials:*

1. Six of of 16-well strips precoated with the F(ab')2 fragments of an anti-thrombomodulin monoclonal antibody (Mab).
2. Dilution buffer: 1 vial of 20 ml dilution buffer, tenfold concentrated. This buffer contained phosphate, bovine albumin, and tween 20. Distilled water ($dH_2O$) was used to make a 1:10 dilution of this concentrated buffer before use.
3. Anti-thrombomodulin peroxidase: 3 vials of immunoconjugate were mixed with 8ml of dilution buffer to reconstitute a vial of anti-thrombomodulin peroxidase conjugate.

4. Reference thrombomodulin; reconstituted with 2ml of dilution buffer to obtain a solution containing 100ng/ml.
5. Washing Solution: 1 vial of 50ml washing solution, 20 fold concentrated. Distilled water was used to prepare a 1:20 dilution to obtain a ready for use washing solution.

6. Hydrogen peroxide.
7. Ortho-Phenylenediamine (OPD) substrate
8. M sulphuric acid:This was obtained by adding 1 volume of
9. concentrated sulphuric acid (18M) to 5 volumes of distilled water.

## *Assay Protocol:*

The reconstituted Reference Thrombomodulin containing 100ng/ml was used to prepare assay calibrators by performing serial dilution (1:2) with dilution buffer.

All plasma to be tested were diluted with 1/5 with dilution buffer. When necessary, a 1/10 dilution were used when samples were suspected to contain an elevated level of thrombomodulin.

## *Assay Procedure:*

*Antigen fixation*: 200 µl of the test sample (calibrator or test plasma) were added to each microwell. After an incubation for 2 hours at room temperature (18 - 25°C), 5 successive washes with washing solution was performed.

*Addition of immunoconjugate*: Immediately after the last washing cycle, 200 µl of immunoconjugate was added to each microwell. Again after an incubation for 2 hours at room temperature, the microwells were successively washed 5 times with the washing solution.

*Colour development*: Immediately after the last wash, 200 µl of OPD and hydrogen peroxide were added to each microwell. After an incubation at room temperature for exactly 8 minutes, the enzymatic reaction was then stopped by adding 50 µl of 3M sulphuric acid to each microwell.

*Plate reading* : The plates were kept at room temperature for 10 minutes for colour stabilization. After that, the optical density of each microwell was read at 492 nm.

*Obtaining results:* A log-log paper was used. The concentration of thrombomodulin calibrators (ng/ml) was plotted on the x-axis, and the corresponding optical density values on the y-axis. The concentrations of thrombomodulin of test dilutions were interpolated on the calibration curve to determine their values. The value obtained was multiplied by the dilution factor (5 or 10) to get the concentration of the tested sample.

## STATISTICAL ANALYSIS

Results were expressed as mean $\pm$ standard error (SEM), Statistical significance was accepted for $p<0.05$. Difference between the time points was calculated using one way analysis of variance. Tukey-HSD test (ANOVA) was used to determine the significance of change in NE in relation to outcome. Spearman rank correlation coefficient test was used to determine correlation.

## **TABLE 3.1**: PATIENTS CHARACTERISTICS

| | |
|---|---|
| Number | 19 |
| Mean Age | 56 $\pm$ 4.5 years (SEM) |
| Sex (M:F) | 7 : 12 |
| Indication for transplantation | |
| Primary biliary cirrhosis | 7 |
| Primary sclerosing cholangitis | 2 |
| Autoimmune hepatitis | 4 |
| Subacute liver failure | 1 |
| Alcoholic liver disease | 5 |

# RESULTS

NE prior to OLT was 40.13 ± 4.84 ng/ml which was significantly higher than those of normal controls (18.7 + 5.6 ng/ml, $p< 0.05$). During the early anhepatic phase, NE levels increased moderately to (71.28 ± 9.11 ng/ml, $p< 0.05$). Following unclamping of portal vein (i.e. reperfusion), there was a marked increase in NE antigen which peaked two hours post-reperfusion (370 ± 50.5 ng/ml, $p< 0.001$). Although there was a gradual decline in the levels of NE, they remained elevated 24 hours after reperfusion (186 ± 60.94 ng/ml) though not statistically significant (fig 3-I).

Two patients died of graft failure on days 7, and 12. A further patient died on day 31 after a stormy postoperative course, which started with initial poor graft function and was later complicated by acute rejection, septicaemia and massive G.I bleeding due to multiple CMV induced ulcers involving different parts of the G.I. tract. The outcome score of these patients ranged between 6-8.

Mean delta NE ( the difference between maximum and base-line values for each patient) correlated with time to prothrombin time (PT) normalization ($p = 0.002$, $r^2 = 0.52$, fig 3-2), but not to alanine transaminase (ALT) levels at 24 hrs post-reperfusion (fig 3-3). Using the criteria of graft outcome which is detailed in chapter two, the mean delta NE was 224.8 ± 36.3 in group I, 442.6 ± 48.5 in group II, and 720 ± 195.8 ng/ml in group III. Only

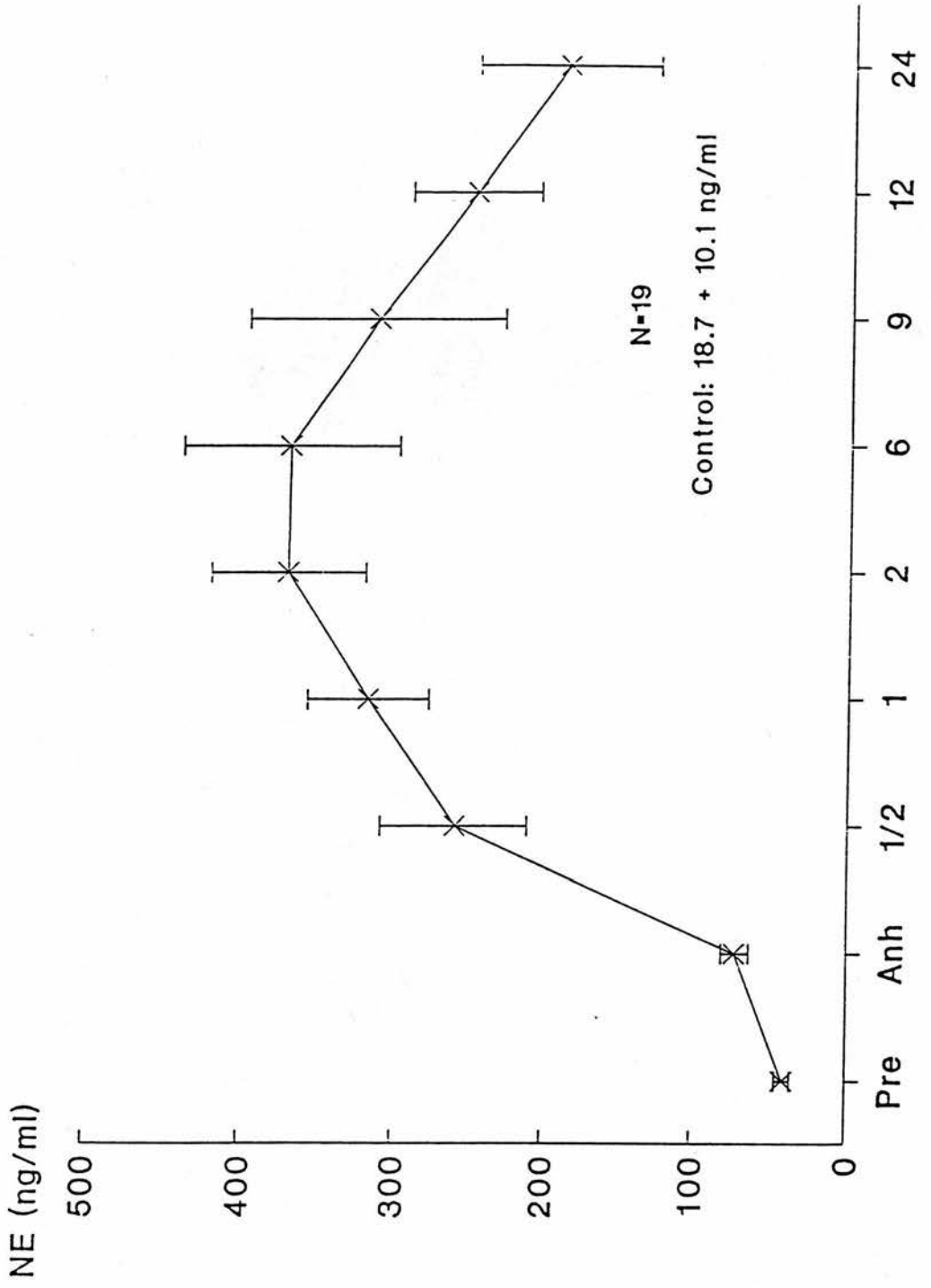



**Figure 3 - 1:** Changes in NE levels before and after reperfusion up to 24 hrs. Pre: before induction of anaesthesia, anh: anhepatic phase

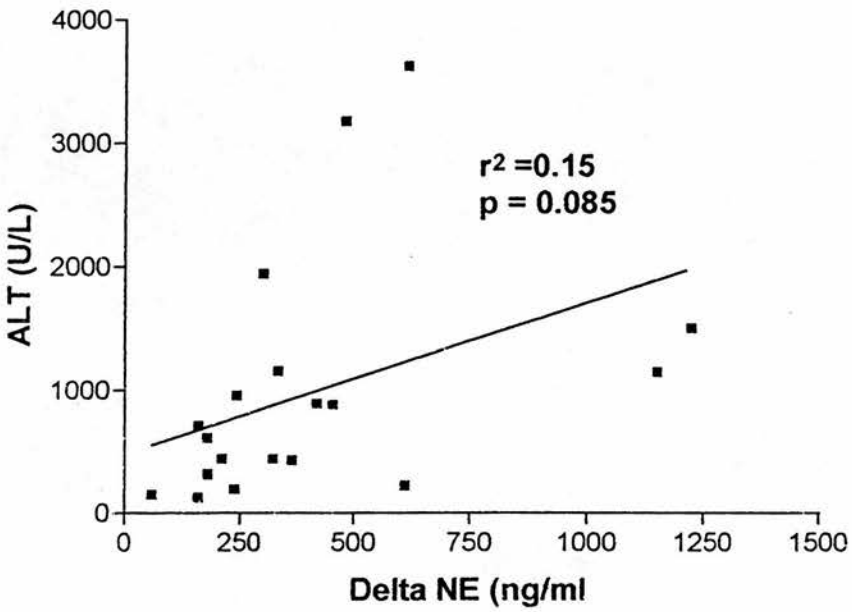



Figure 3-3: This graph demonstrates the lack of correlation between mean changes in neutrophil elastase (NE) and alanine transaminase (ALT) 24 hrs post-reperfusion.

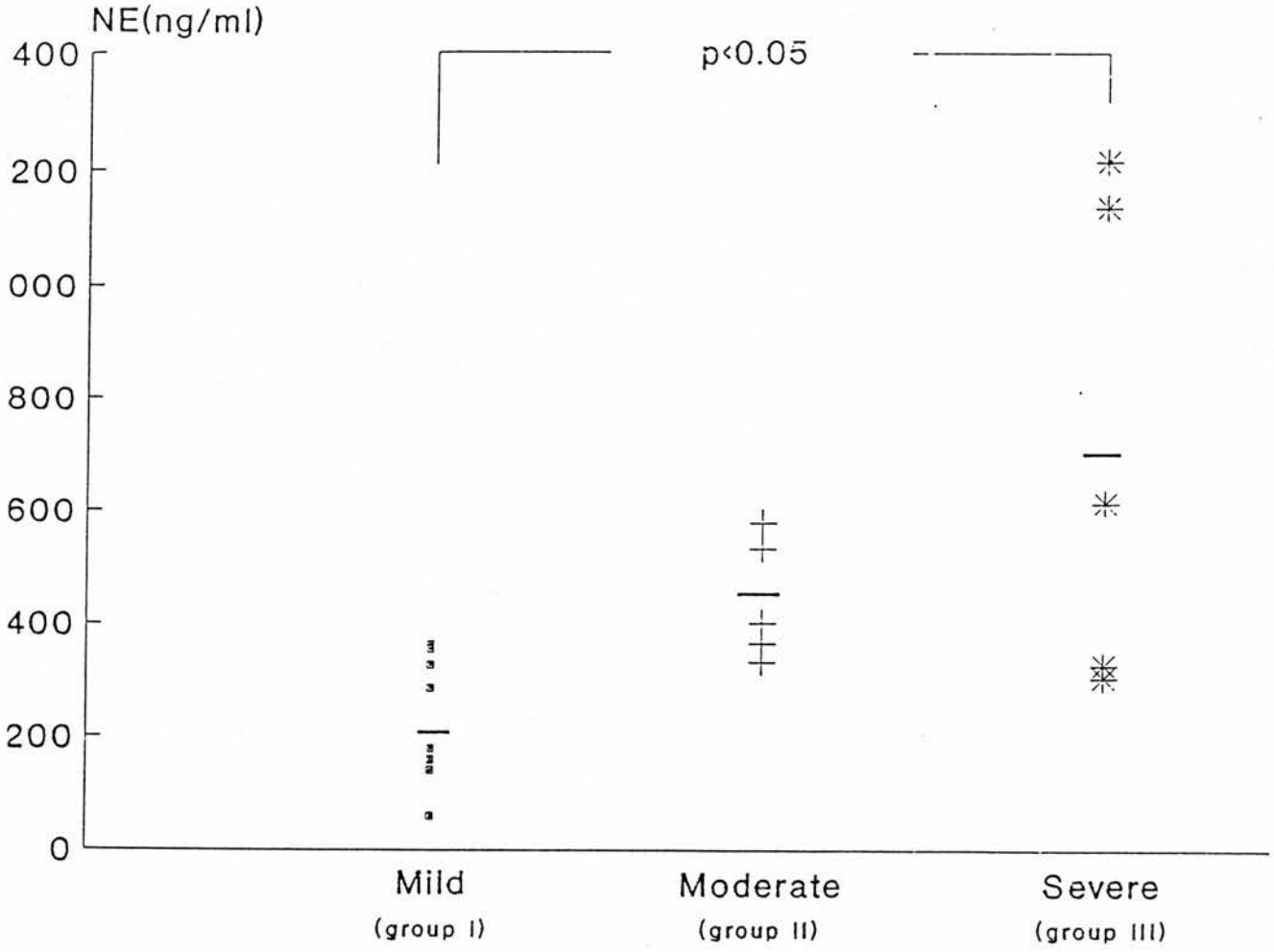



**Figure 3 - 4:** Changes in delta NE in relation to graft outcome (patients classified into three groups according to their total outcome score, using the criteria in table 2-2): group I, mild graft dysfunction with a total score up to 3; group II, moderate dysfunction, with a score of 4-6; and group III, severe dysfunction, with a score of 7-9. A total score of up to 6 is usually associated with good outcome.

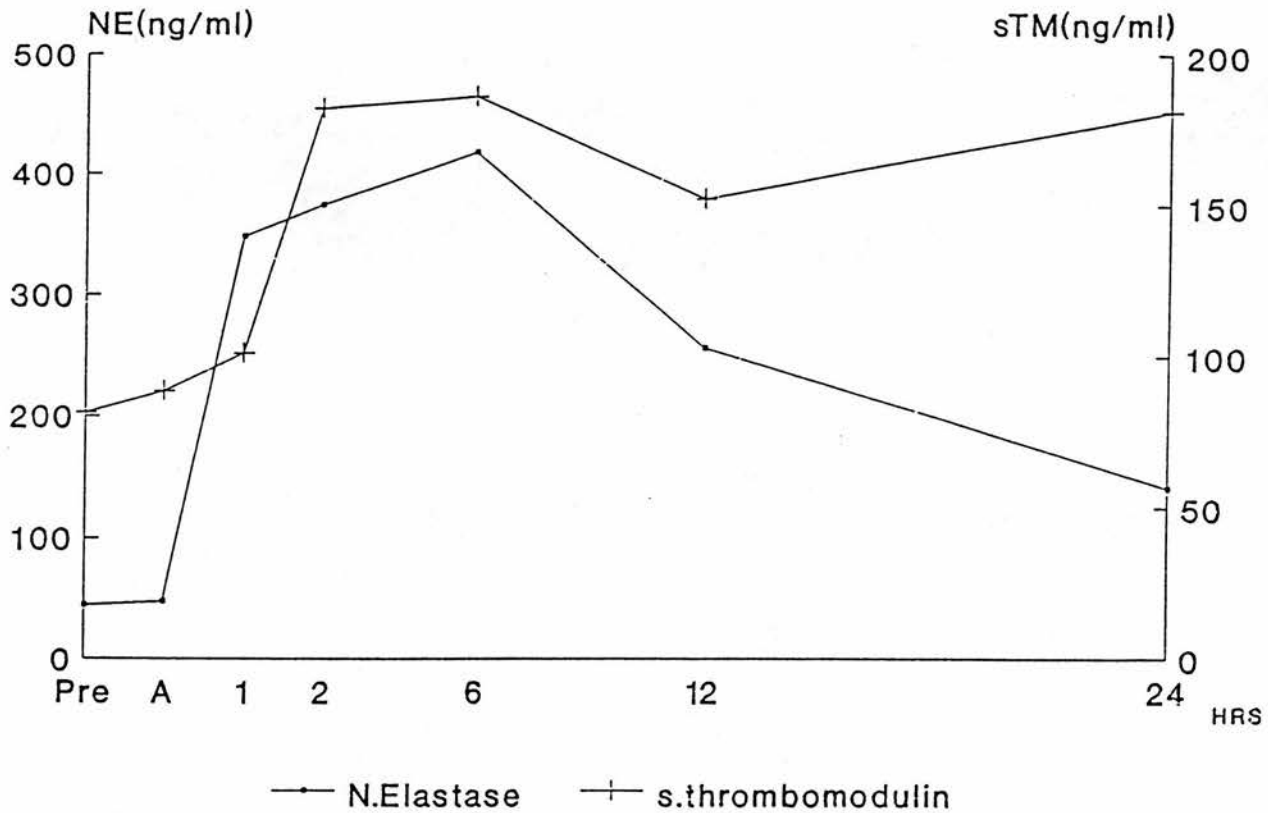



**Figure 3-5:** Changes in sTM and NE levels before transplantation (pre), during the anhepatic phase (A), and after reperfusion up to 24 hrs.

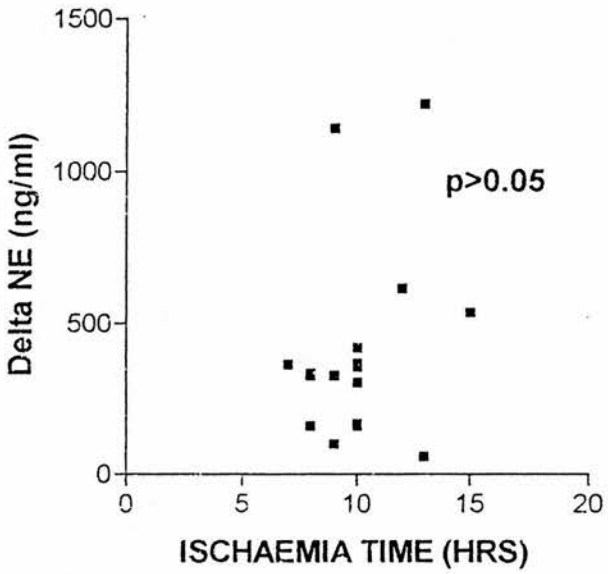



Figure 3-6: This graph shows the relationship between cold ischaemia time and the mean changes in neutrophil elastase (NE) perioperatively

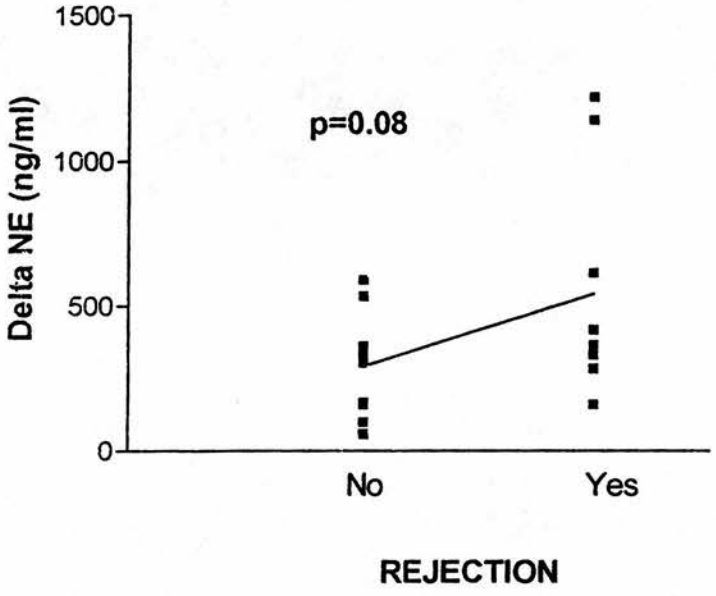



Figure 3-7: This graph demonstrates that the mean increase in neutrophil elastase (NE) perioperatively is not significantly different in patients with or without rejection.

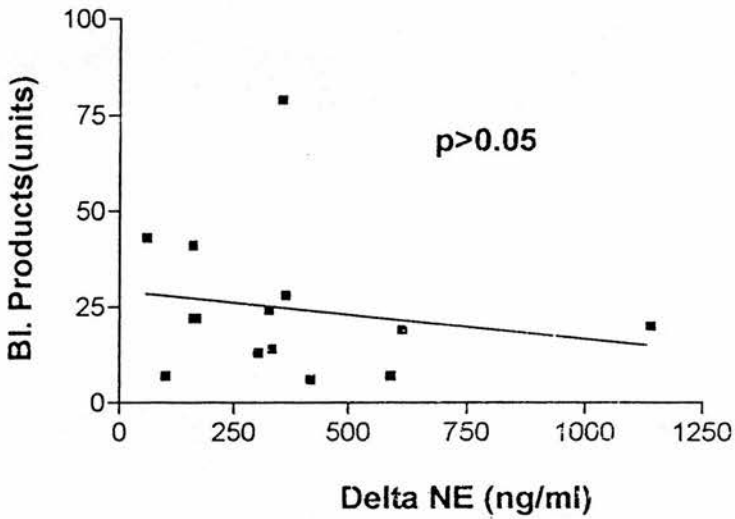



Figure 3-8:Correlation of the changes in neutrophil elastase (NE) activity and the total amount of blood and blood products administered peroperatively (NS).

# DISCUSSION

Histological studies demonstrating infiltration of neutrophils in tissues which were exposed to transient ischaemia followed by reperfusion support the notion that neutrophils contribute to reperfusion injury (Engler et al 1986). Once released from neutrophils, NE causes direct damage to intact cells and inactivates various proteins such as immunoglobulins, complements, and clotting factors (Weiss, 1989). Further, NE facilitates increased generation of free radicals by converting xanthine dehydrogenase into oxidase in the vascular endothelial cells (Phan et al, 1992). Such effect would set the scene for a vicious cycle by which free radicals in addition to their direct tissue damage would contribute to further neutrophil chemmoattraction, rolling, and adhesion via enhancing expression of pro-inflammatory mediators such as adhesion molecules (e.g GMP-140) platelet activating factor (PAF) and the leukotriene LTB4 (Lewis et al 1988, Lehr et al 1991, Patel et al 1991). Free radicals also inhibits neutralizing NE by its specific inhibitor $\alpha_1$.antitrypsin via oxidation of methionine at position Met-358 (Janoff, 1985).

We have shown that the baseline values of NE in the patients studied were higher than those of a healthy volunteer population. This is in keeping with the observations of previous studies from our group, and others which demonstrated evidence of neutrophil activation in patients

with chronic liver diseases, particularly those with an alcoholic etiology (Hayes et al 1989, Antonsen et al 1988).

The level of NE increased markedly following reperfusion which was sustained for several hours thereafter. The relatively short duration between the samples taken during anhepatic phase and early post-reperfusion further suggests that neutrophil activation rather than for instance delayed renal or hepatic clearance is the likely mechanism of such changes.

Changes in NE activity post-reperfusion were significantly higher in patients with poor graft function using the criteria described shown in table-I. Patients in group II showed higher mean NE compared with those of group I, however the changes did not reach statistical significance. The highest values were seen in two of the three patients who died post-operatively as a consequence of poor graft function. Such findings would further suggest a significant role played by neutrophils in reperfusion injury

The degree of rise in NE levels following reperfusion seen in this study was markedly higher than that detected in conditions such as acute myocardial infarction, multiple trauma, or inflammatory bowel diseases (Bell et al 1990, Donnelly et al 1992, Gionchetti et al 1994). This might indicate a more significant neutrophilic damage following liver graft reperfusion when compared with the above mentioned conditions. The fact

that we collected our samples much more central and closer to the likely site of increased activity in order to minimize the effects of dilution could have played a significant role in such differences.

Although VVB could potentially cause neutrophil activation, we do not feel that this has played a significant role in the changes seen in this study, as the most marked increase in NE levels occurred well after VVB was discontinued, and remained so for several hours thereafter. In some patients, significantly high levels of NE were seen 24 hours following the discontinuation of VVB. In addition the continuous infusion of aprotinin which inhibits platelet aggregation and formation of NE-$\alpha$1 antitrypsin complex during the VVB reduces neutrophil activation further (Wachtfogel et al, 1993).

In this study sTM increased significantly following reperfusion, which is comparable to those of a recent study in patients who had OLT (Sido et al 1995). There was a trend of earlier rise in NE to be followed shortly with rapid rise of sTM. Further a strong correlation was found between the mean perioperative changes of sTM with those of NE, which suggests an endothelial cell damage as a result of increased proteolytic activity of neutrophils following graft reperfusion in this group of patients.

The nonthrombogenic property of endothelial cells is important in maintaining proper blood circulation. Components of the nonthrombogenic

mechanism include two major well-characterised anticoagulant systems that operate on the endothelial cell surface: the TM-thrombin-protein C system (Esmon et al, 1982), and the glycosaminoglycan-antithrombin III system (Rosenberg et al, 1984). The physiologic relevance of the TM-thrombin-protein C system is emphasised by the occurrence of thrombosis in patients with a congenital deficiency of protein C (Comp, 1986). Numerous reports have demonstrated that inflammatory mediators from activated neutrophils damage the barrier function and the integrity of endothelial cells. Loss of the anticoagulant property of the endothelial surface in such a way in liver transplantation potentially leads to microcirculatory stagnation and tissue ischaemia and this might be an important mechanism behind the onset of no-reflow phenomenon, a recognized feature of preservation-reperfusion injury.

## Conclusion

This study demonstrated a significant degree of neutrophil activation following graft reperfusion during OLT, with evidence of endothelial cell damage as a result of such activity. As early neutrophil recruitment and graft thrombosis appear to play a major role in graft failure following prolonged preservation, additional strategies to interfere with neutrophil endothelial interactions and procoagulant mechanisms are likely to be of potential clinical utility.

# CHAPTER FOUR

## INDOCYANINE GREEN CLEARANCE REFLECTS REPERFUSION INJURY FOLLOWING LIVER TRANSPLANTATION AND IS AN EARLY PREDICTOR OF GRAFT FUNCTION

# INTRODUCTION

Graft dysfunction could result in prolonged ITU care, increased risk of sepsis, and in severe cases (PNF) a need for early retransplantation. Early diagnosis is vital for appropriate action. Reliable tests, however, which permit early diagnosis of this condition are not currently available. Liver function tests are difficult to interpret in the early post-operative period and the diagnosis at present relies on a combination of biochemical, haemodynamic and clinical markers (Forester et al 1989, Chazouilleres et al 1993)

Greig et al (1989) have proposed a four stage classification of primary graft dysfunction based on transaminase activity, bile production and coagulopathy but serial observations up to 72 hours are required for their scoring system. In addition, bile flow is no longer easily measured as most transplant centres do not routinely insert a biliary T-tube. We have previously shown in a preliminary report that indocyanine clearance measured on the day after liver transplantation accurately reflected graft function and may be used to predict graft survival and final outcome (Jalan et al 1994).

Indocyanine green is a water soluble tricarbocyanine dye which is extracted by hepatic parenchymal cells and excreted almost entirely into bile. It does not undergo any enterohepatic circulation (Wheeler et al, 1958) . The concentration in the plasma can be easily measured by spectrophotometry and its rate of elimination has been widely used as a measure of liver blood flow and liver function (Caesar et al, 1961) . In patients with chronic liver disease, ICG clearance is probably the preferred method of expressing the rate of elimination as the volume of distribution is highly variable (Gilmore et al, 1982) .

## AIMS

The aim of this study was to further evaluate ICG clearance as a predictor of outcome and to test the hypothesis that ICG clearance following orthotopic liver transplantation reflects the degree of reperfusion injury.

# METHODS

The ICG clearance is an already existing and established clinical test. As such, there was no need for ethical approval and this was discussed with the ethical subcommittee.

**Patients & Transplant Details:**

Peripheral ICG clearance was measured in 41 consecutive patients (46 transplants), between 18 and 24 hours after liver transplantation. Details of the patients included are summarized in table 4-1.

The first 24 transplants were used to determine the cut-off value of ICG clearance which best predicts graft outcome . This has been found to be 200 ml/min, and was reported in a preliminary study (Jalan et al 1994). The subsequent 22 transplants were used to further evaluate this cut-off value for the prediction of graft function.

The postoperative data recorded were:

Haemodynamic stability.

Time to correction of acidosis.

Time to normalization of prothrombin time.

Correction of encephalopathy.

Liver function tests.

# METHODOLOGY

### (a) ICG clearance

ICG clearance was measured using standard methodology (Gilmore et al, 1982). Sterile freeze dried indocyanine green (Paeselain Lorei, Biochemika, Frankfurt) was dissolved immediately prior to injection in the aqueous solvent provided to the concentration of 2.5 mg/ml. A dose of 0.5 mg/kg of body weight was injected rapidly into a central vein after a baseline plasma sample was taken. Samples were repeated at 5 minute intervals from the time of injection for 30 minutes.

Plasma was separated by centrifugation (3,000 rpm for 15 minutes). ICG concentration was measured by spectrophotometry. This was calculated by reading the absorbency of plasma samples at 805 nm; background turbidity was corrected by measuring the absorbency at 900 nm. The absorbency was converted to a concentration expressed as mg/l from a standard curve. The concentration was subsequently plotted on semi-logarithm paper against time. The line of best fit was drawn using the least squares method. Clearance was calculated as the product of the elimination rate constant K multiplied by the volume of distribution. The volume of distribution was calculated as the ratio of the dose to the extrapolated concentration at time 0 while the elimination rate constant K

was the ratio of the logarithm to the graphically read half life. The coefficient of variation was 4% .

**(b) Reactive Oxygen Intermediates (ROIs)**

ROIs were measured in 24 patients using the diene conjugated linoleic acid method. The percentage ratio of diene conjugated linoleic acid (PL-9, II-LA) to its substrate phospholipase esterified linoleic acid (PL-9, 12-LA) was used as a determinant of free radical activity. Details of the method used are included in chapter two.

A blood sample was taken just before the operation (baseline sample) and subsequent samples were taken during the anhepatic, reperfusion phase and at 6-hourly intervals for 24hrs. The maximum rise of ROI (maximum Diene/Linoleic acid ratio minus the pre-operative (baseline) value) was recorded for each patient.

**(c) Neutrophil Elastase (NE)**

NE was measured by radioimmunoassay using rabbit polyclonal antiserum (14). Details of the assay are included in chapter four. Results were expressed in ng/ml. The sensitivity of the assay was 0.5 ng/ml. The normal range was between 20-51 ng/ml. Blood samples were taken before the operation (baseline) and during the anhepatic, reperfusion phase and at 6,

12 and 24 hrs. The maximum rise in NE (maximum NE concentration minus pre-operative "baseline" value) was recorded for each patient .

## STATISTICAL ANALYSIS

A receiver operator curve (ROC) was used to determine the best cut-off value to predict outcome, using the sensitivity and specificity of several cut off values of ICG clearance. The Pearson correlation coefficient was used to correlate ICG clearance with a number of biochemical parameters postoperatively and with reactive oxygen intermediates and neutrophil elastase levels; a p value at a level of 5% was taken as statistically significant (two-tail test of significance). All values were expressed as Mean ± SEM.

**TABLE 4.1: PATIENTS CHARACTERISTICS**

| | |
|---|---|
| Number | 41 (46 transplants) |
| Mean Age | 49 ± 2 (SEM) years |
| Sex (M:F) | 15 : 26 |
| Indication for transplantation | |
| Primary biliary cirrhosis | 14 |
| Primary sclerosing cholangitits | 5 |
| Autoimmune hepatitis | 2 |
| Fulminant liver failure | 9 |
| Paracetamol | (6) |
| Non-paracetamol | (3) |
| Cryptogenic cirrhosis | 5 |
| $2^{ry}$ sclerosing cholangitis | 1 |
| Hepatocellular carcinoma | 1 |
| Alcoholic liver disease | 4 |

# RESULTS

In all patients, ICG clearance was performed between 18 to 24 hours following transplantation. None of the patients was actively bleeding or clinically septic at the time of the test. All but seven patients were still ventilated and 8 patients required inotropic support. Duplex colour flow Doppler and ultrasonography was performed at day 1 and confirmed normal hepatic arterial and portal venous flow in all patients.

At 24 hrs post-transplantation, the mean ICG clearance was 347.3 ± 17.8 ml/min, ALT l389 ± 188 u/1, bilirubin l29 ± 12 μmol/l and prothrombin time 26 ± 2 sec. The mean time to correction of acidosis and prothrombin time were 1.2 ± 0.1 and 3.2 ± 0.4 days respectively.

In figure 4-1, the data for all 46 transplants is presented. 37 out of 38 patients with ICG clearance above 200 ml/ min. were discharged home within 30 days of the transplant and well with no problems due to graft function. One patient with paracetamol induced fulminant failure required prolonged ITU support and was discharged home well after 2 1/2 months.

Eight patients had a clearance below 200 ml/min. of whom six had either died or required re-transplantation (four retransplanted for primary graft non-function from whom two died 4 and 7 days later, two died at 12 and 31

days post-transplantation). Two survived after prolonged stay in ITU and intensive ventilatory and renal support. The highest sensitivity and specificity of ICG clearance as a test to predict outcome was at cut-off values of 200 & 250 ml/ min (100% sensitivity, 95% specificity) (figure 4-2).

When ICG clearance was plotted against ROIs, there was a significant negative correlation between maximum rise of ROI activity and ICG clearance indicating that the higher the oxygen radical activity which is a mediator of reperfusion injury, the lower the ICG clearance ($r = -0.64$, $p < 0.0008$) (figure 4-3).

When ICG clearance was plotted against maximum rise of NE, a significant negative correlation between those two parameters was found, the higher the neutrophil elastase, the lower the ICG clearance (r - -0.65, $p < 0.0005$) (figure 4-4).

ICG clearance significantly correlated with the ALT activity ($r = -0.35$, $p<0.02$), and prothrombin time ($r= -0.40$, $p<O.OO77$) at 24 hrs post-transplantation (figures 4-5, and 4-6 respectively). However neither ALT nor prothrombin time at day 1 were found to be sensitive and specific enough to predict outcome. No correlation was found between ICG clearance and serum bilirubin ($r = -0,26$, $p=0.08$, figure 4-7) , hydrogen ion concentration at 24 hrs post-transplantation ($r = - 0.05$, $p= O.7$), time to

correction of prothrombin time ($r = -0.20$, $p=0.2$) or time to correction of acidosis ($r = -0.19$, $p= 0.24$).

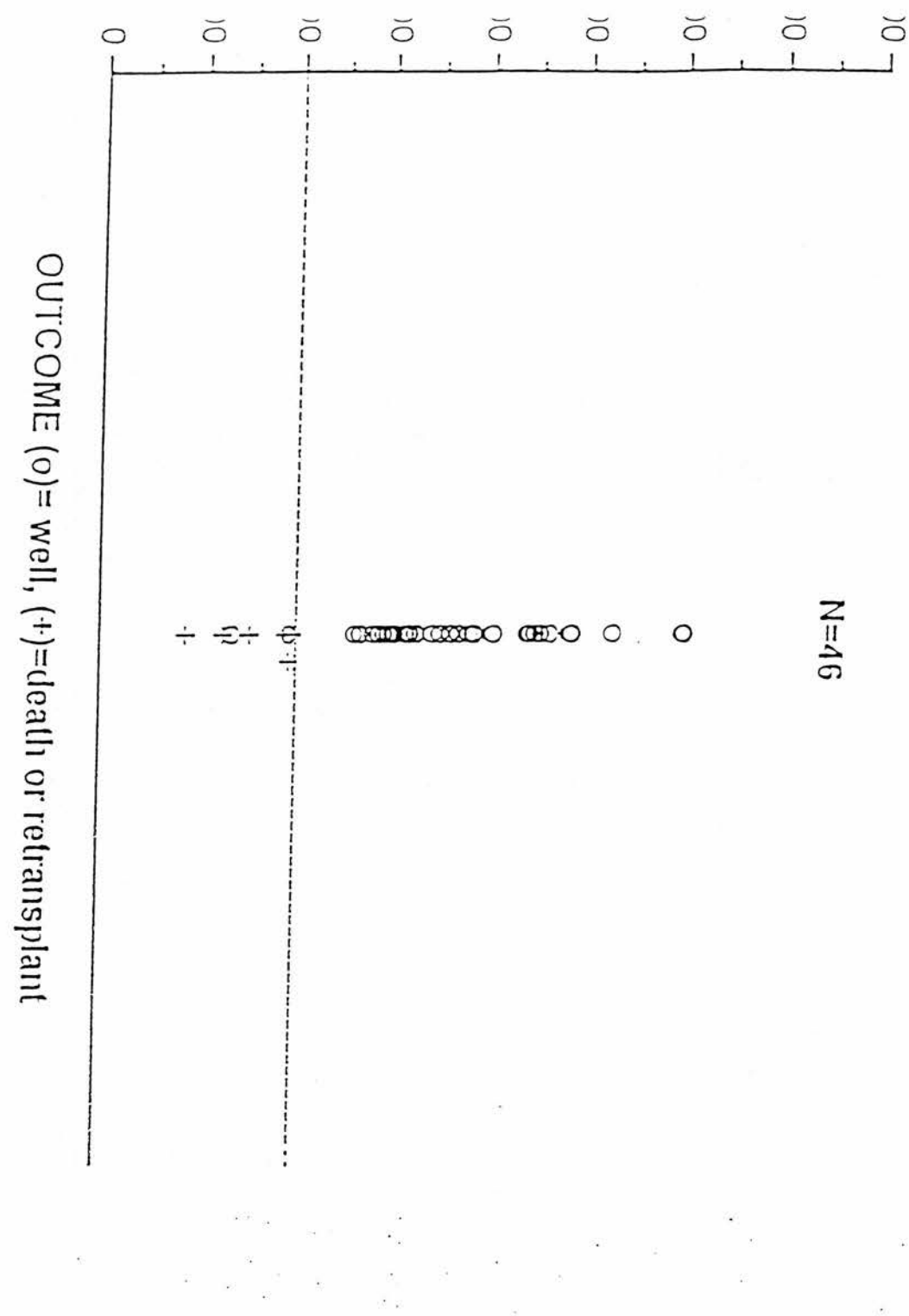



**Figure 4-1**: ICG clearance as a function of outcome following liver transplantation

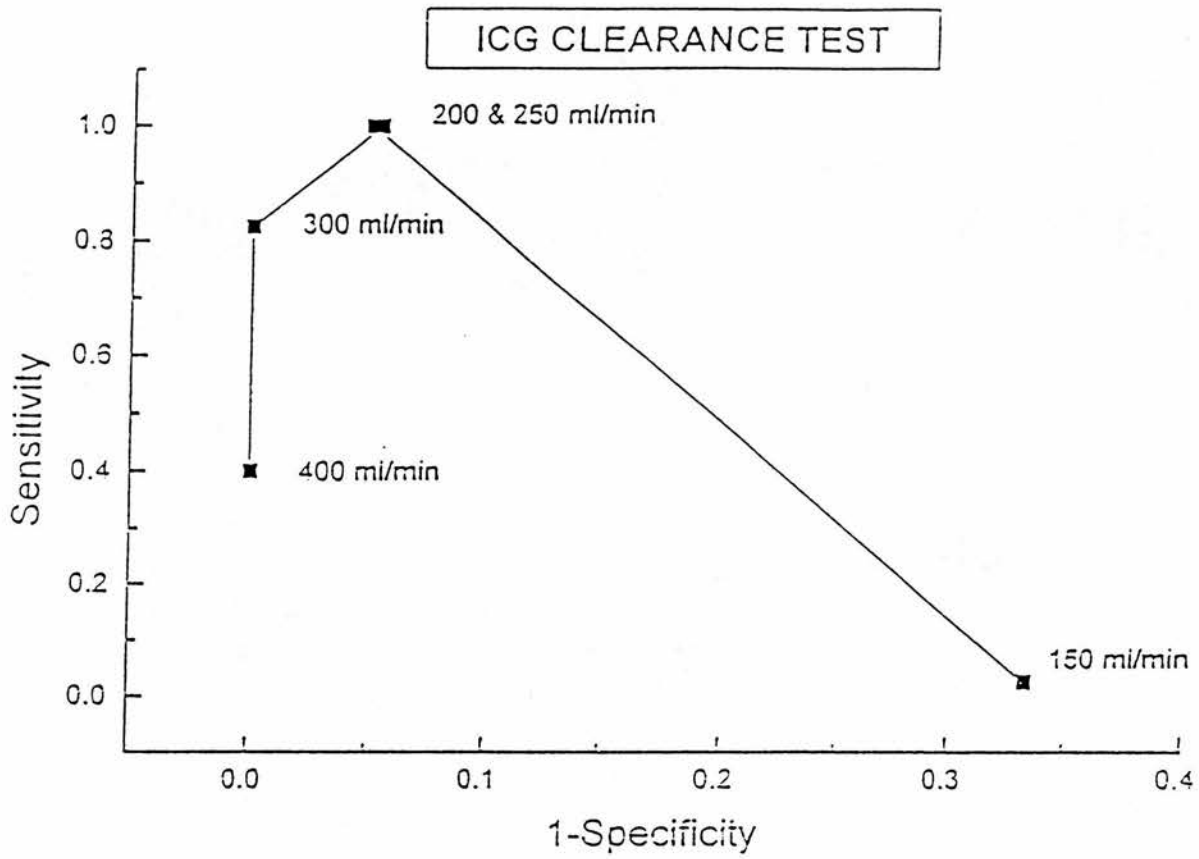



Figure 4-2: ROC curve of 4 cut-off values of ICG clearance test as a predictor of outcome.

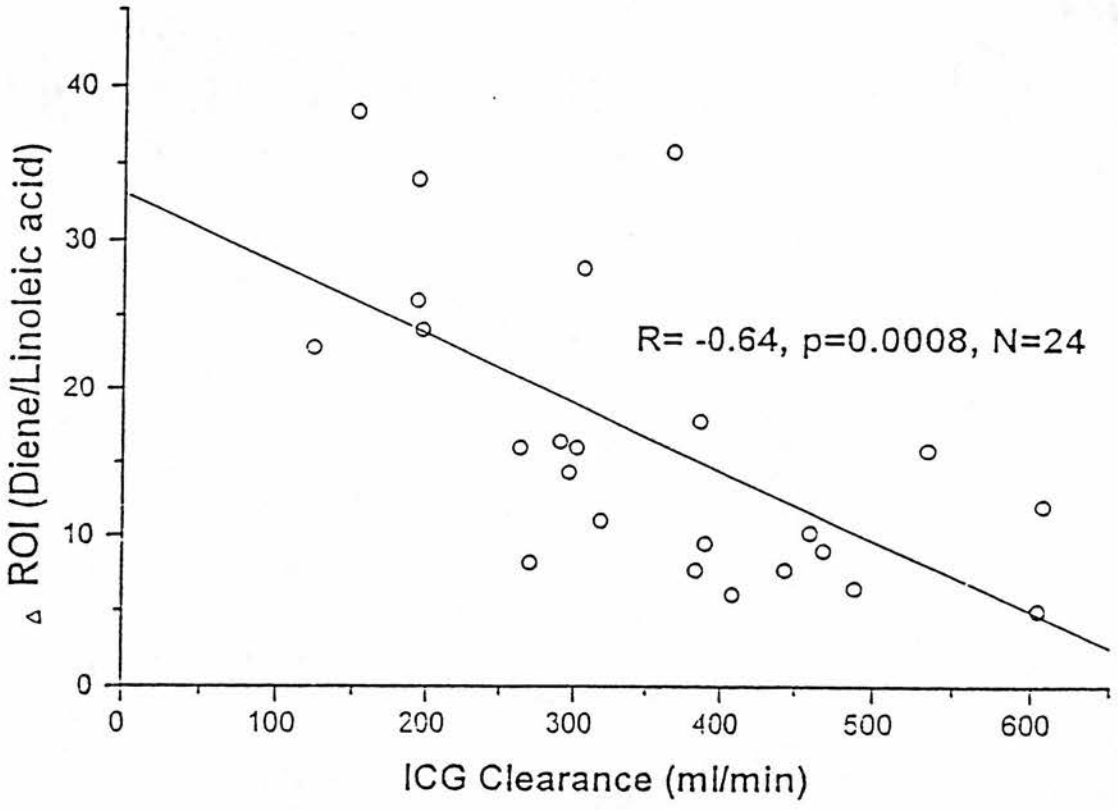



Figure 4-3: Relationship between ICG clearance within 24 hrs post-transplantation and maximum rise of reactive oxygen intermediates (ROI) perioperatively; a statistically significant inverse correlation was found

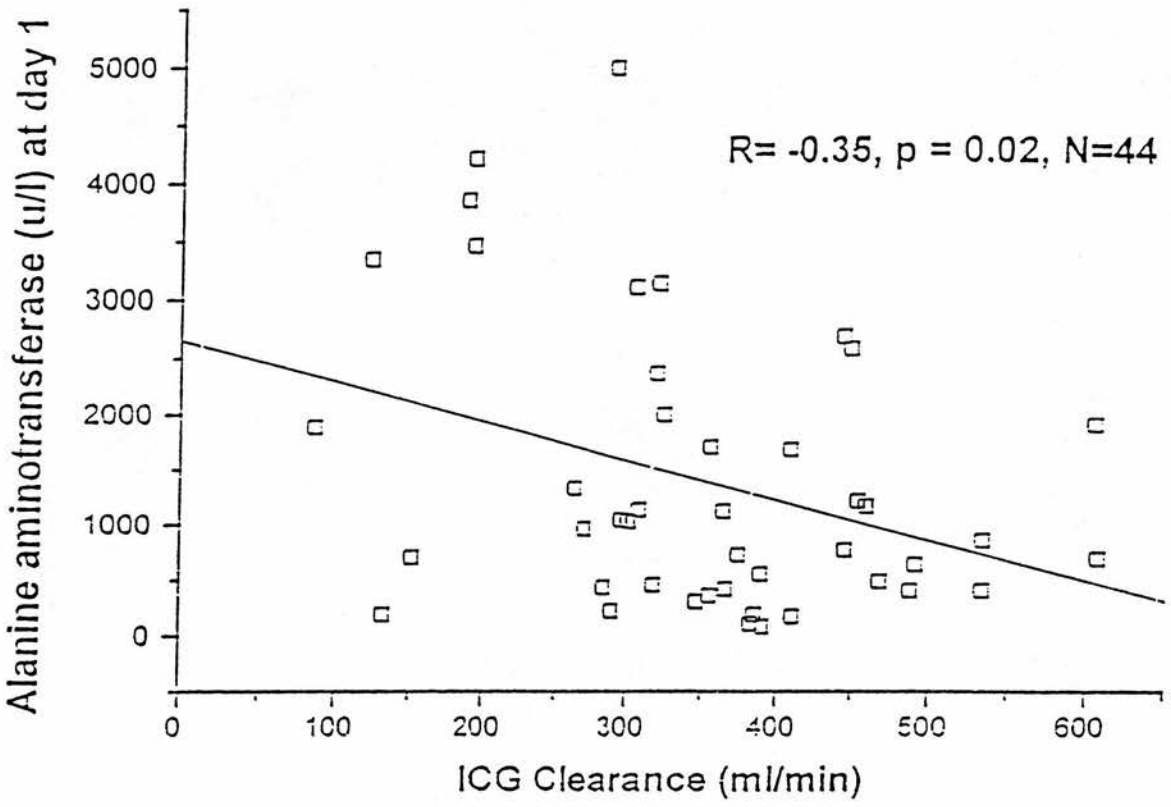



Figure 4-5: This graph illustrates the correlation between ICG clearance and alanine aminotransferase at 24 hrs.

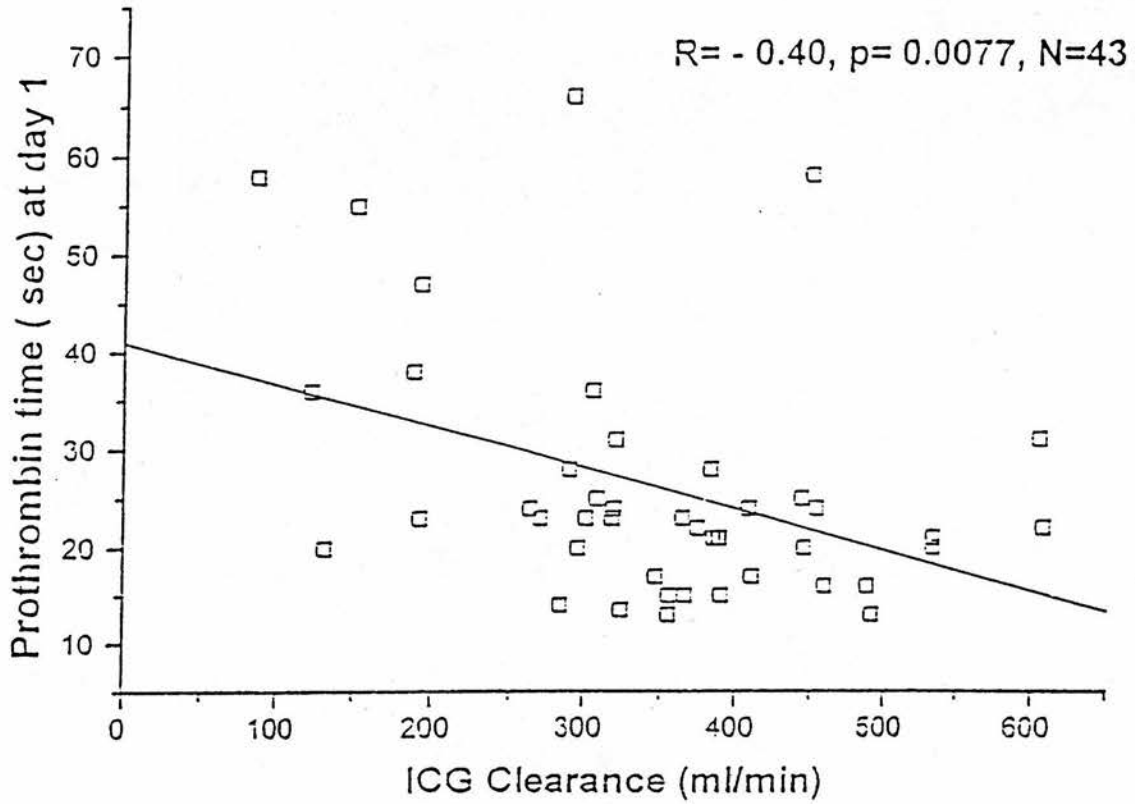



Figure 4-6: This graph illustrates the correlation between ICG clearance and prothrombin time at 24 hrs.

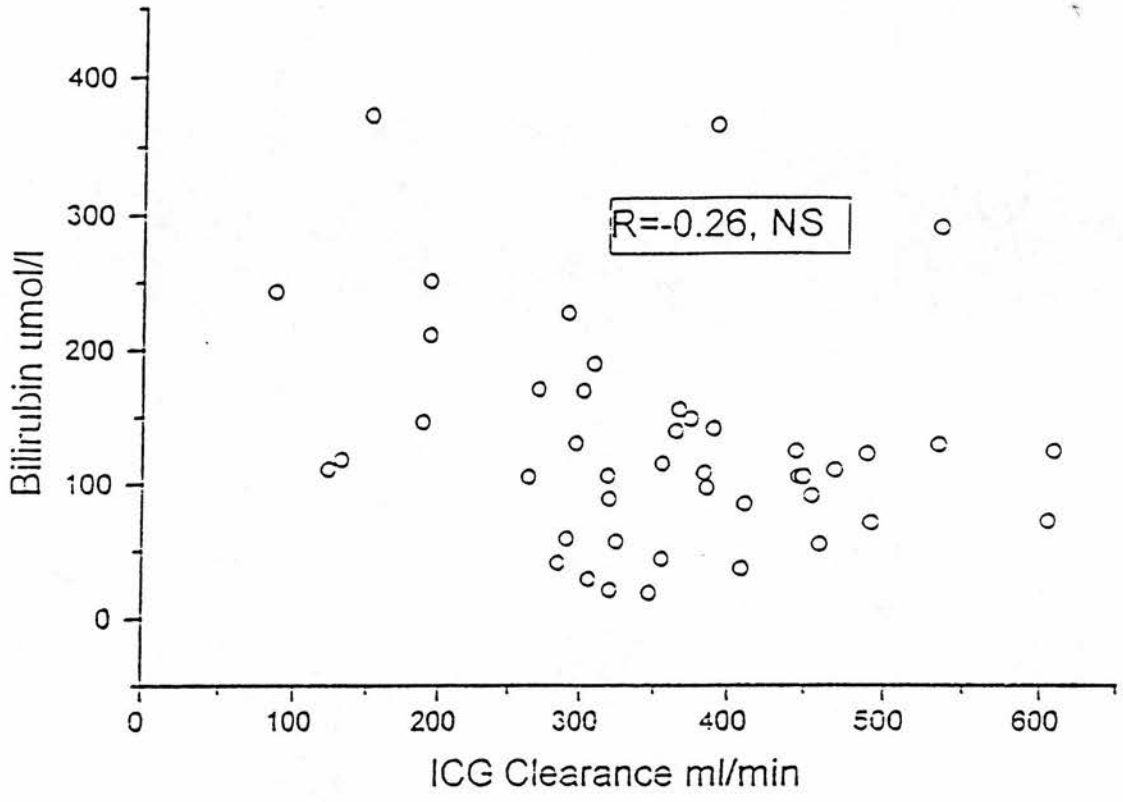



Figure 4-7: This graph illustrates the of correlation between ICG clearance and bilirubin level at 24 hrs.

# DISCUSSION

Primary graft dysfunction when serious is a life-threatening condition and early diagnosis is critical. Recently several groups have attempted to devise a classification of primary graft dysfunction either based on the combination of clinical and biochemical parameters (Greig et al 1989, Gonzalez et al 1994) from both donor and recipient data or by using dynamic liver function tests including galactose or caffeine (Nagel et al 1990, Svensson et al 1989) but none has been adopted into routine clinical practice. Measurement of ICG clearance appears to be a simple and safe test to assess early liver graft function. It can be easily measured by spectrophotometry and the development of non-invasive techniques such as Near-Infra Red Magnetic Spectroscopy (NIMRS) (1B-20) has enabled bedside assessment of the elimination of ICG with immediate results thereby increasing its clinical usefulness.

Although ICG clearance has been traditionally thought to reflect liver blood flow, recent studies using NIMRS technology by Shinohara et al (1996) have demonstrated that the elimination of ICG by the rabbit liver follows two exponential rate constants; the first reflects the dye uptake from plasma to hepatocytes and the second represents the dye removal from the liver by cytoplasmic transport and biliary excretion. The latter process

can be inhibited by the presence of excess bilirubin. In primary graft dysfunction it is probable that both the mechanisms of uptake as well as removal of the dye are affected.

In the present study we have shown that ICG clearance is the most specific and sensitive single test to predict graft function and outcome in the immediate post-operative period thus enabling an earlier consideration of retransplantation for graft failure. In our study group, a clearance of both 200 and 250 ml/min had the same sensitivity and specificity in predicting outcome, but we opted to use the lowest threshold (200 ml/ min) as a cut off value for significant graft dysfunction since we had two patients who survived despite a clearance below 200 ml/ min.

All patients had normal hepatic artery and portal vein blood flow post-transplant, therefore the most likely cause of deranged uptake from plasma to hepatocytes appears to be a change in the liver microcirculation. Changes in the microcirculation as a result of sinusoidal endothelial damage and Kupffer cell activation secondary to reperfusion injury are common (Koo et al 1992), this being a result of accumulation and enhanced endothelial adhesion of neutrophils (Engler et al 1986), increased platelet aggregation and adherence (Kubes et al 1990) and the release of a number of mediators such as reactive oxygen intermediates (Parks et al 1983, Arthus 1988, Bzeizi et al 1993), neutrophil elastase

(Janoff, 1985, Phan 1992, Bzeizi et al 1996), leukotrienes (Lehr et al 1991) and endothelin-II (Goto et al 1994). These phenomena are likely to exert a direct effect on delivering oxygen to the hepatocytes thereby decreasing their function which may be reflected by reducing their ability to excrete indocyanine green. In our study high serum bilirubin did not appear to have any significant inhibitory effect on excretion of indocyanine green because no correlation of day-II post-operative bilirubin level and ICG clearance was found. This favors the hypothesis that a reduced ICG clearance is mainly due to impaired microcirculation and compromised hepatocyte function.

In this study we have chosen to measure two markers of reperfusion injury namely reactive oxygen intermediates (ROIs) and neutrophil elastase (NE) and found that those markers correlate well with ICG clearance reinforcing the hypothesis that ICG clearance reflects the degree of reperfusion injury. We therefore propose that ICG clearance reflects graft function and can be used routinely in the evaluation of primary graft dysfunction in the immediate post-operative period. Although the routinely used haemodynamic and biochemical parameters, are clearly useful in the evaluation of the clinical condition of the patient, they are misleading in the early post-operative period because several factors, such as sepsis, derangement of fluid and electrolyte balance, transfusion of blood products and degree of renal dysfunction, can make their interpretation difficult.

In conclusion, this study has shown that an ICG clearance <200 ml/min measured within 24 hrs following liver transplantation predicted graft dysfunction due to reperfusion injury with high specificity and sensitivity. ICG clearance is a relatively cheap and easy to perform test with immediate results. The use of NIMRS technology can simplify the measurement of ICG clearance and may increase further its usefulness further by permitting frequent bedside monitoring of the clearance (Hopton et al 1995, Shinohara et al 1996).

# CHAPTER FIVE

## INFLUENCE OF cGMP CHANGES ON THE HAEMODYNAMICS FOLLOWING REPERFUSION IN LIVER TRANSPLANTATION.

# INTRODUCTION

The reperfusion phase during orthotopic liver transplantation (OLT) is a critical event with well recognized haemodynamic and cardiac changes which sometimes could be profound and may result in intra-operative death (Lichtor, 1991). The haemodynamic changes, defined as post-reperfusion syndrome are predominantly an increase in pulmonary capillary wedge pressure (PCWP) and central venous pressure (CVP), along with a reduction in mean arterial pressure (MAP) and systemic vascular resistance (SVR). Such haemodynamic derangements tend to improve in the majority of patients within 30-60 minutes, requiring only cardio-vascular pharmacological support. However, the changes may be severe enough in a small proportion of patients to be life threatening (Aggarwal, 1989).

Several potential mechanisms for these changes have been considered including sudden influx of cold, acidic and hyperkalaemic blood, embolization, or release of vasoactive substances from the grafted liver (Marino et al 1985). Ischaemia and reperfusion which accompany organ transplantation is associated with release of pro-inflammatory mediators, generation of free radicals along with neutrophil sequestration and

activation (Bulkely, 1994). Such mediators could influence endothelial cell function leading to disturbance of vasomotor control.

Free radicals have been shown to counter-act the relaxation effects of nitric oxide (NO), on vascular smooth muscle cells via inhibition of guanylyl cyclase and reduction in the release of guanosine 3`, 5`-cyclic mono-phosphate (cGMP) from endothelial cells (Cherry et al, 1990). We have demonstrated earlier evidence of an enhanced free radical activity post-reperfusion in liver transplantation which leads to the suggestion that cGMP generation could be reduced as a result of that, and hence impaired vascular smooth muscle relaxation.

Endothelins play an improtant role in vasular tone control (Masaki et al 1991), with their potent vasoconstrictor actions and they might influence the haemodynamic changes during transplantation. Studies have shown an increase in the levels of endothelins following ischaemia-reperfusion. Lerman et al (1991) showed an increase in circulating endothelins immediately after liver transplantation and Textor et al (1992) demonstrated that the increased endothelins after liver transplantation had a profound effect on the renal haemodynamics.

They are released from the vascular endothelial cells to act on specific endothelin (ET) receptors which are abundant predominantly in the renal

and pulmonary vascular beds (Shibuta et al 1990). Endothelins caused Profound increase in PVR in rat lung transplantation following ischaemia-reperfusion.

## AIMS:

This study was designed to investigate the potential role of cGMP and endothelins in the haemodynamic changes during liver transplantation,

# PATIENTS & METHODS

***Patients Details:-***

Fourteen patients (6 males and 8 females) with a mean age of 52.4 ± 5.4 (SEM) years were studied. The indication for transplantation was:

| | Patients |
|---|---|
| Primary biliary cirrhosis. | 6 |
| Alcoholic liver disease. | 5 |
| Cryptogenic cirrhosis. | 3 |

All patients underwent veno-venous bypass during the anhepatic phase of the transplant procedure, with a mean duration of 71.5 ± 9.3 minutes. Detailed haemodynamic measurements were recorded after induction of anaesthesia, during the anhepatic phase and following reperfusion at 30, 60 and 120 minutes.

***The haemodynamic measurements were:***

Pulmonary arterial pressure (PAP)

Pulmonary capillary wedge pressure (PCWP)

Both, were determined using a Swan Ganz catheter.

Mean arterial pressure (MAP): continuously measured using an intra-arterial line.

Cardiac output (C.O): obtained in triplicate using the thermodilution technique.

Systemic vascular resistance (SVR): calculated, using the standard equations, SVR(dynes/sec/cm$^5$) = (MAP - CVP) x 79.9/C.O.

pulmonary vascular resistance: PVR(dynes/sec/cm$^5$) = (PAP - PCWP) x 79.9/C.O.

We also recorded the amount of fluid administered to maintain haemodynamic stability, and the blood products used to maintain haemoglobin concentration > 10 gm/dl, platelet count > 50 x $10^9$/L, and prothrombin time < 16 seconds.

***Blood samples collection:***

Blood samples were collected into EDTA containing tubes from a catheter placed at the right atrium, at the same time points as the haemo-dynamic recordings. Plasma was separated immediately (centrifuged for 15 minutes, at 3000 g) and stored at -70°C until analysis. The plasma samples were later used for measurement of cGMP and endothelins. The cGMP was measured by an in-house radioimmunoassay using rabbit antibody RIBG and ED2-3 respectively. Endothelins (ET-1, &ET-2) were

measured by radioimmunoassay using $^{125}$I-ET-A (Amersham, London, UK). Both assays are described in detail in the following part of this section.

## cGMP MEASUREMENTS

The cyclic nucleotide (cAMP and cGMP) concentration in each sample was measured using an in-house radioimmunoassay (Millat et al., 1993). This method is based upon that described by Brooker et al. (1979), and uses rabbit antisera produced in response to an acetylated form of the appropriate cyclic nucleotide. Consequently, the sensitivity of the assay is increased by acetylating the nucleotides in both test samples and standards. In our laboratories this assay gives inter (on 10 samples)and intra assay (3 samples) coefficients of variation of 3.5% and 4.2%, respectively.

### Materials

*Assay buffer:*

40 mls 0.5M Acetic Acid (BDH Chemicals Ltd.Poole,England)

60 mls 0.5M Sodium Acetate (BDH Chemicals Ltd.Poole,England)

0.1% BSA (Sigma Chemical Company, St. Louis, USA) added to 900 mls distilled water.

*Acetylating mix:*

Trichioroethylamine (TEA) and Acetic Anhydride in a ratio of 2:1 (Sigma Chemical Company, St. Louis, USA).

*Dextran coated charcoal solution:*

22.6g Na HPO (BDH Chemicals LTD Poole, England), 5.44g KH P0 (BDH Chemicals LTD Poole, England), 2g NaN (Sigma Chemical Company, St. Louis, USA), 12g charcoal (Sigina Chemical Company, St. Louis, USA) l.2g dextran (Pharmacia Uppsala, Sweden), and 0.8g gelatin (Sigma Chemical Company, St. Louis,USA)

**Summary of the Method:**

Cyclic nucleotides were extracted by mixing 500μl of plasma using 2 volumes (1ml) of 100% ethanol, followed by cooling at 4°C (15min). The samples were then spun at 3, 000rpm at 4°C (15min) and the supernatant containing the extracted cyclic nucleotides, was decanted into fresh tubes. The samples were subsequently dried under air (50°C) and resuspended in 500ml assay buffer (0.05M acetic acid; 0.05M sodium acetate; 0.1% gelatin; pH 4.8).

For the radioimmunoassay, a standard curve was prepared covering the range 320-0.03nM. Each standard (500μl) and each sample (500μl ) was acetylated using 15ml of a mixture containing triethylamine (2 vols) and acetic anhydride (1 vol). Duplicate samples (100μl ) of each standard and each sample were taken and both an $^{125}$I-cyclic nucleotide trace (150μl / 4, 000cpm) and the relevant, appropriately diluted, antiserum (100μl ) were

added. The samples were mixed thoroughly and allowed to equilibrate at 4°C (16-20 hours). Following equilibration, 600µl dextran coated charcoal were added to each tube, and these were centrifuged at 3, 000rpm (15 min). The supernatant was separated from the charcoal and discarded, and the contents of each tube was counted in a gamma counter. The cyclic nucleotide content of each sample was calculated by interpolation from the standard curve.

## ENDOTHELINS MEASUREMENTS:

### *Principles of the assay:*

Amersham's endothelin-1,2 (high sensitivity) [$^{125}$I] assay system was used for quantitative determination of endothelin-1 (ET-1), ET-2 and big ET-1. The system utilizes a high specific activity [$^{125}$I] endothelin-1 (synthetic) tracer, together with a highly specific and sensitive antiserum. The assay is based on the competition between unlabelled ET-1 and a fixed quantity of [$^{125}$I] labelled ET-1(synthetic) for a limited number of binding sites on an ET-1 specific antibody. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand.

### *Materials:*

- Assay buffer concentrate (10 ml). On dilution to 100 ml, this will give 0.02M borate buffer $P^H$7.4 containing 0.1% sodium azide.
- Endothelin-1(synthetic), lyophilized, Upon reconstitution, the vial contained 320fmol/ml ET-1.
- Rabbit anti-endothelin serum.
- Tracer as $^{125}$I-ET-A (synthetic) -41kBq, 1.1μCi, lyophilized.
- Amerlex-M second antibody reagent. Donkey anti-rabbit serum coated on to magnetizable polymer particles. This solution contained 0.06% w/v sodium azide

***Specimen Collection And Sample Preparation:***

Blood samples were taken from an indwelling catheter placed in the right atrium and collected in tubes containing EDTA. The time points of the samples taken were identical to those for cGMP sampling and corresponding to the haemodynamic recordings used in this study. Plasma was separated immediately (centrifuged at 3000g for 15 minute, at 4 °C) and was stored at –40°C until analysis.

***Extraction Procedures:***

1. The columns were equilibrated by washing with 2ml methanol followed by 2ml of water.

2. Plasma (1ml) was acidified with 0.25ml 2M HCl, centrifuged at 10000g for 5 min at room temperature and loaded onto the column. This was followed by washing with 5 ml water and 0.1% trifluoroacetic acid(TFA).
3. Further washing with 2 ml 80% methanol in water + 0.1% TFA. The eluent was collected in a glass tube, and later dried down in a centrifugal evaporator.

***Radioimmunoassay Procedure:***

1. 2.0 ml of the assay buffer was added to the standard to make a solution contains of ET-1 (synthetic) at a concentration of 320 fmol/ml in 0.02M borate buffer.
2. 11 ml of the assay buffer was added to the antiserum and another 11ml to the tracer to make solutions containing antiendothelin serum in 0.02M borate buffer, and [$^{125}$I]ET-1 (synthetic) in 0.02M borate buffer respectively.
3. 500 μl of assay buffer was added onto each of the polypropylene tubes labelled 0.25, 0.5, 1, 2, 4, 8, and 16. Into the 16 tube, 500 μl of the assay standard was added (320 fmol/ml), and vortexed thoroughly.
4. 500 μl were transferred from tube 16 to the 8 tube to make a double dilution. The doubling dilution procedure was then repeated successively with the remaining tubes.

5. 100 μl aliquots from each serial dilution along with 100 μl aliquots of 320 fmol/ml standard solution gave rise to 8 standard levels of ET-1 ranging from 0.25-32 fmol (0.623-79.74pg).

***Assay Protocol:***

1. Reagents and assay standards were prepared as described above. and were equilibrated to room temperature.
2. Polypropylene tubes were labelled in dublicate for total counts (TC), non-specific binding (NSB), zero standard ($B_0$), standards and samples.
3. 200 μl of assay buffer were pipetted into the NSB tubes, and 100 μl into $B_0$ tubes.
4. 100 μl of each standard and 100 μl of the unknown sample were pipetted into the appropriately labeled tubes. 100 μl of 320 fm/l standard were added into tube labeled 32.
5. 100 μl of the antiserum were added into all the tubes except NSB and TC. All the tubes were mixed thoroughly, covered with a plastic film and incubated for 4 hours at 2-8 °C.
6. 100 μl of [$^{125}$I]ET-1 were added into all tubes, followed by thorough vortex mixing and incubation for 16-24 hours at 2-8 °C.
7. 250 μl of Amerlex-M second antibody reagent were added into each tube except the TC and incubated for 10 minutes.

8. The antibody bound fraction of each tube was separated by centrifugation at 4°C for 10 minutes at 3000g. After centrifugation, the supernatant was discarded, and the tubes were inverted and allowed to drain for 5 min.

9. The radioactivity present in each tube was determined by counting for 60 seconds in a gamma scintillation counter.

10. Calculation of results: The average count per minute (cpm) was calculated for each set of replicate tubes. The percent $B_0$/TC, and the percent bound/ $B_0$ (for each standard and sample) were calculated using standard formulae.

11.The standard curve was generated by plotting the percent $B/B_0$ (a function of the log ET-1 concentration) on the y-axis against fmol standard/tube in the x-axis. The fmol/tube was read directly from the graph.

**Statistical Analysis:**

All results were expressed as mean ± standard error (SE). Difference between mean before and after reperfusion was determined by one way analysis of variance. Correlation between variables was determined using Spearmann rank correlation coefficient. Values were considered as statistically significant when the *p* value was less than 0.05.

# RESULTS

Changes in the haemodynamic parameters are summarized in table 5-I. The main changes were of PVR and PAP. The PVR increased slightly during the anhepatic phase from a base line value of 62.8 ± 12.9 to 72.5 ± 9.6 dyn/s/cm$^5$ (NS). More marked changes were seen following reperfusion with PVR increased to 135 ± 42.7 dyn/s/cm$^5$ ($p< 0.01$), and PAP from 17 ± 1.2 to 22.8 ± 2.6 mmHg, ($p< 0.05$), 30 minutes after reperfusion. The latter increased to a maximum of 23.5 ± 1.9 mmHg, 2 hours after reperfusion.

C.O decreased slightly but not significantly during the anhepatic phase to 8.8 ± 0.78 L/min from mean pre-transplant value of 10.3 ± 2.4 L/min. MAP decreased significantly at 30 minutes post-reperfusion ($p<0.05$) with spontaneous improvement and return to base-line values subsequently.

The mean pre-transplant level of cGMP was 5.33 ± 0.7 ng/ml (fig 5-1) which was significantly higher than those of healthy volunteers (1.35 ± 0.43 ng/ml, n = 10, $p<0.01$). cGMP increased marginally during the anhepatic phase to 6.31 ± 0.76 ng/ml (NS) but following reperfusion there was rapid and significant decrease in cGMP to 1.63 ± 0.50 ng/ml ($p<0.01$).

The mean pre-transplant level of endothelins was 28.75 ± 1.45 pg/ml (fig 5-2), significantly higher than those of healthy volunteers matched for age (15.4 ± 0.8 pg/ml, $p$ <0.01). During anhepatic phase , ET activity decreased to 26.09 ± 1.81 pg/ml, however the changes were not statistically significant. Post-reperfusion, ET values remained comparable to those obtained during the anhepatic phase (26.92 ± 1.38, and 25.08 ± 1.45 pg/ml at one and two hours respectively).

The base-line cGMP correlated inversely with SVR, and MAP ($p<0.05$, and $p = 0.023$ respectively) and directly with PVR recorded at the same time point ($p< 0.05$). The correlation however was more obvious between the mean changes in post-reperfusion cGMP and those of PVR ($r = 0.7$, $p = 0.01$, $n = 12$) and PAP ($r = 0.74$, $p = 0.005$, $n = 12$), (figures 5 - 3 & 5 - 4). No correlation was detected between the intraoperative cGMP changes and those of CVP, and CO at any time point.

Mean blood transfusion requirements were 7.3 ± 4.2 units (range: 2-18), cryoprecipitates 3.6 ± 1.5 units (0-16), platelets 5.2 ± 1.3 units (0-10), and fresh frozen plasma 7.5 ± 2.1 units (2-12). No correlation was found between these parameters and the changes in cGMP or the endothelins.

All patients received dopamine at a fixed infusion rate of 2 μg/kg/min. Seven patients required noradrenaline (8 mg%) at a rate of 4.5 ± 1.2 ml/hr, and 4 of these patients also required adrenaline (1:100,000) at a rate of 3 ± 0.5 ml/hr. Mean post reperfusion changes in cGMP was 2.9 ± 0.7 ng/ml, in these patients and 2.7 ± 0.65 ng/ml in patients who did not require any support (NS).

**TABLE-I**

**CHANGES IN HAEMODYNAMIC PARAMETERS DURING ORTHOTOPIC LIVER TRANSPLANTATION**

| TIME | MAP (mmHg) | CVP (mmHg) | CO (L/min) | SVR (dyn/s/cm$^5$) | PAP (mmHg) | PVR (dyn/s/cm$^5$) |
|---|---|---|---|---|---|---|
| **PRE-ANHEPATIC** | 88.5±1.67 | 6±0.6 | 10.3±1.7 | 633±106 | 17±1.21 | 62.8±12.9 |
| **ANHEPATIC** | 87±2.07 | 6.5±0.86 | 8.8±0.78 | 731±67.8 | 16.8±0.92 | 72.5±9.6 |
| **POST-REPERFUSION** | | | | | | |
| **30 minutes** | 74±2.47 | 6.2±0.9 | 9.6±1.3 | 571±76 | 22.8±2.6* | 135±42.7** |
| **60 minutes** | 97±.51.75 | 7.5±0.7 | 9.3±1.29 | 612±61.3 | 23.1±2.4* | 111.7±30.2* |
| **120 minutes** | 82±2.13 | 8.5±0.6 | 8.8±1.0 | 660±66.6 | 23.5±1.9* | 106.8±14.6* |

*: $p<0.05$, **: $p<0.01$

MAP: mean arterial pressure, CVP: central venous pressure, C.O: cardiac output, SVR&PVR: systemic &pulmonary vascular resistance, PAP: pulmonary arterial pressure

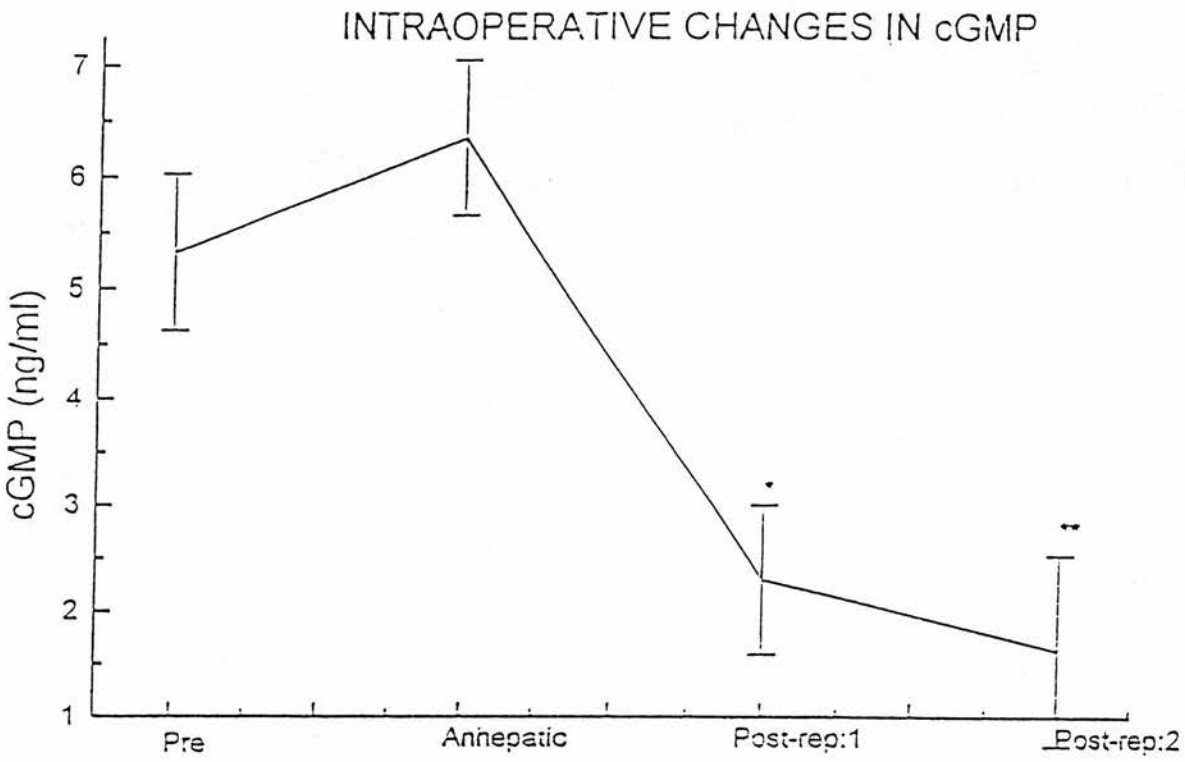



Figure 5-I: The perioperative profile of cGMP. Pre = pre-anhepatic, Anh = anhepatic, and then post-reperfusion at 30, 60, 120 minutes.

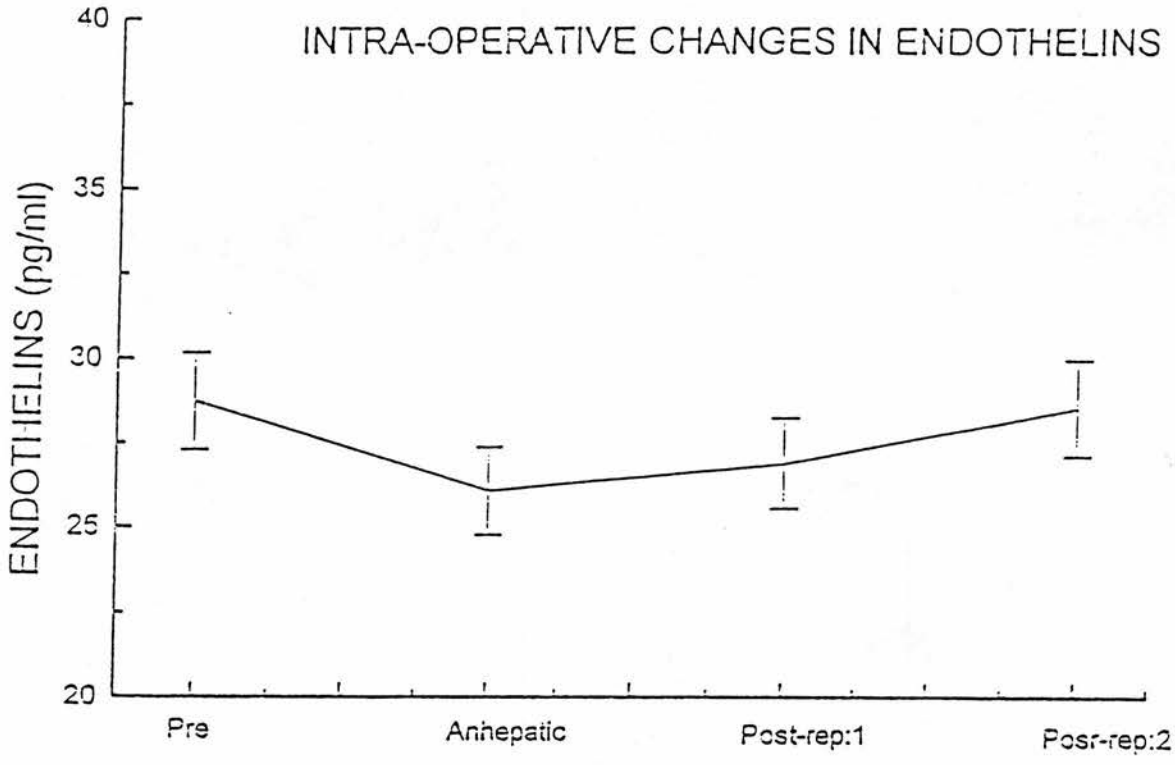



Figure 5-2: The perioperative profile of endothelins. Pre = pre-anhepatic, Post-rep:1= post-reperfusion at 60 minutes, and post-rep:2 = post-reperfusion at 120 minutes.

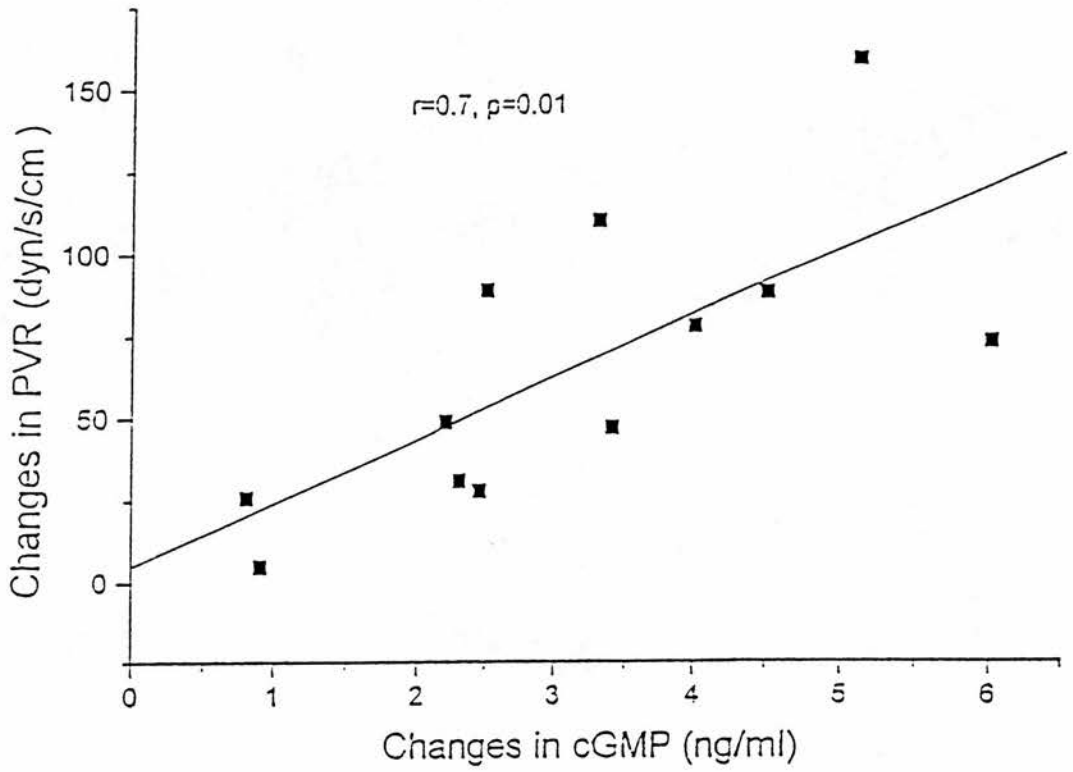



Figure 5-3: Correlation of the changes in cGMP (difference between pretransplant value and minimum post-reperfusion one for each patient) with those of pulmonary vascular resistance (PVR).

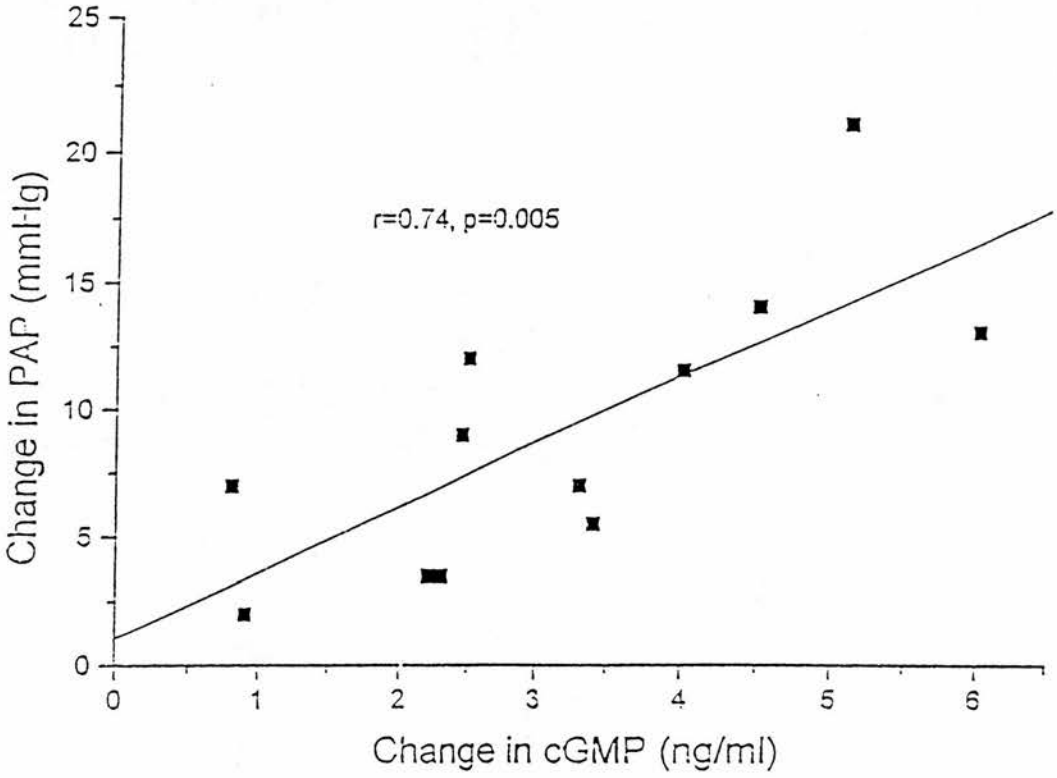



Figure 5-4: Correlation of the changes in cGMP with the those of pulmonary arterial pressure (PAP).

# DISCUSSION

This study confirms the previous observations of major haemodynamic disturbances during OLT (Kang et al 1987). The most striking changes were in PVR and PAP; both of which rose shortly after reperfusion. The changes in PVR and PAP correlated with the changes in cGMP levels.

cGMP, the vascular smooth muscle cell relaxant (Cherrry et al 1990), was elevated in this group of patients with end stage chronic liver disease prior to transplantation. Reduction in its concentration after reperfusion could be a result of decreased activity of its main stimulants, or perhaps it could represent mere normalization. Whichever the mechanism behind such changes, the reduction in cGMP levels after reperfusion of the graft and the correlation with the changes in the pulmonary haemodynamic parameters suggest a causal relationship.

Although, levels of cGMP continued to fall after reperfusion, PVR showed signs of recovery. There is no direct explanation to these changes . One possibility could be the release of other vasomotor mediators that could interfere with the actions of cGMP. Prolongation of the haemodynamic readings and measurements of cGMP for several hours afterwards may help in delineating this relationship further.

Our findings of elevated pre-transplant cGMP are comparable to the previous reports of elevated levels of NO, the main stimulant of cGMP generation in patients with cirrhosis Cahill et al 1995, Claria et al 1994). Niederberger et al (1995), have shown in a cirrhotic animal model an increase in aortic cGMP which was reduced significantly following inhibition of NO production by the NO synthase inhibitor $N^G$-monomethyl-L-arginine (L-NMMA). This reduction in NO followed by reversal of the hyperdynamic abnormalities of the systemic circulation. Recently, Sogni et al (Sogni et al 1995), showed a significant correlation between endogenous NO and pulmonary vasodilatation seen in cirrhosis. Addition of the cGMP analog 8-(4-chlorophenylthio)-guanosine-3',5'-cGMP to the preservation fluid in an experimental rat model of lung transplantation lead to significant reduction of PVR and an increase in pulmonary artery blood flow after reperfusion (Pinsky et al 1994). These findings further support our conclusion about the role of cGMP in the haemodynamic alterations observed during transplantation.

Although cGMP may be generated by pathways that do not involve NO, such as stimulation of membrane associated guanylyl cyclase by atrial natriuretic peptide (ANP), or by enterotoxins, NO is the major stimulant for cGMP release from the soluble guanylyl cyclase (Kelly et al 1992). cGMP is released by virtually all cell types through activation of guanylyl cyclase.

cGMP levels are regulated by its rate of synthesis by guanylyl cyclase and its rate of hydrolysis by cyclic nucleotide phosphodiestrase (Murad, 1986), and its half life ranges between 10-20 minutes (Voderwinker 1991).

The release and effects of cGMP and NO, could be influenced by free radicals which are generated following ischaemia-reperfusion. NO is the only known biological molecule that can be produced in high enough concentration to outcompete superoxide dismutase (SOD) for radicals. This interaction results in formation of peroxynitrite anion ($ONOO^{\cdot}$), a radical with vasomotor properties unlike those of its parent compound NO (Darley-Usmar et al, 1994). Superoxide anion has been shown to induce vasoconstriction, and is known to have a direct inhibitory effect on guanylyl cycalse leading to a decrease in cGMP production (Cherry et al, 1990).

Pinsky et al (1994), studied in-vitro the interaction of NO-cGMP with free radical activity during ischaemia-reperfusion, as detailed earlier in chapter one. They showed a reduction of NO production by vascular endothelial cells exposed to hypoxia followed by reoxygenation with a consequent loss of the ability to inhibit thrombin-induced platelet aggregation. addition of superoxide dismutase to the reaction mixture largely restored the ability of reoxygenated endothelial cells to inhibit thrombin-induced platelet aggregation. These findings support the hypothesis that NO reacts rapidly with superoxide, generated by reoxygenated endothelial cells or within the

vasculature during reperfusion of an organ (forming peroxynitrite in the process). As a result, not enough NO is available to activate guanylate cyclase. The findings in our study of decreased cGMP levels post-reperfusion are supported by those of Pinsky et al, and it makes it plausible to consider that free radicals could have played a role in the changes of cGMP observed in our study.

Base-line endothelins were higher than those of age matched healthy volunteers. These findings are comparable to the previously reported results in patients with cirrhosis (Uchihara et al 1992). Mean values of ETs during anhepatic phase and post-reperfusion were not significantly different from the base-line ones. We could not identify any correlation between ETs measurements and any of the haemodynamic parameters of the similar time points.

Despite the the lack of any significant correlation between the endothelins and the haemodynamic alterations observed in this study, it is however still possible that endothelins could have played a role. One possibility perhaps is the imbalance in vasomotor control caused by the rapid reduction in the vasorelaxing effects of cGMP.

In human liver transplantation as compared to experimental animal studies, several factors could influence the overall levels of humoral

mediators measured from plasma or serum. These include the haemodilutions effects of perioperative blood and fluid administration, as well as the role of veno-venous bypass(VVB). In this study we have collected our samples through a catheter placed in the right atrium, a point in proximity to the effluent blood from the liver in order to minimize the effects of dilution. We have also determined in detail the fluid and blood requirements throughout the operation, and none of these correlated with endothelins measurements.

In summary, this study demonstrates haemodynamic changes affecting predominantly the pulmonary vasculature following reperfusion of liver graft during OLT. These changes correlated significantly with a reduction in the levels of cGMP, the mediator of vascular smooth muscle relaxation, which indicates a causal relationship. It also suggests that endothelins do not change significantly post-reperfusion and are unlikely to contribute to post-reperfusion syndrome. Further studies looking at the relationship of cGMP and NO with the other mediators which rise following graft reperfusion will be of major interest. These will provide the pathophysiological basis behind therapeutic options of maintaining cardiovascular stability during the critical phase of reperfusion in organ transplantation.

# CHAPTER SIX - DISCUSSION

# DISCUSSION

**Prediction and Recognition of Primary Non-Function:**

The technical advances in the field of liver transplantation including the developments of preservation fluids, operation techniques and the intra-, and post-operative anaesthetic and ITU care have all contributed significantly to the progress and widespread application of transplantation in the management of acute and chronic liver disease. Despite these advances however, the reported incidence of primary graft dysfunction has not changed significantly. Predicting and avoiding PNF, the severe form of graft dysfunction remains a challenge. Unfortunately, PNF can also become a challenge to diagnose when it does occur. Distinguishing between the initial poor function of a graft that will eventually recover (PGD), and one that is displaying PNF is not an easy task. Thus, there have been many efforts both to predict and to recognize PNF as early as possible in the transplantation process. Perhaps the most obvious means by which to assess and predict graft function is to perform a biopsy of the organ. Several studies have examined the use of frozen section biopsies to predict graft outcome without success except in the presence of the most severe pathological changes (D'Alessandro 1991, Adam 1990, and Markin et al 1990). To illustrate the frustrating nature of PNF further, one has only to appreciate the impressive number of parameters that have

been investigated in efforts to identify its risk factors. Makowka et al (1987) retrospectively analyzed a variety of commonly used donor criteria in an attempt to predict outcome after transplantation. They found these criteria to be completely unreliable and, most interestingly, found that more than one half of donors judged to be "poor" provided organs with good early function. Subsequent reports have made similar observations (Greig 1990, and Kakizoe et al 1990). It would also be satisfying to identify some particular laboratory value or test that might be predictive in allograft outcome. Again, a multitude of parameters have been investigated. Preoperative serum creatinine (Cuervas-Mons et al 1986), enzyme levels in the perfusate of stored organs (Iu et al 1987), hydrogen clearance rates as an assessment of microcirculation (Manner et al 1990), liver tissue oxygenation at the time of reperfusion (Goto et al 1992), platelet adherence within the graft (Cywes et al 1993), post-transplantation thrombocytopenia (McCaughan et al 1992), and intraoperative measures of multiple variables (Paulsen et al 1989) have all been advocated as the best predictors of early graft function. Unfortunately none are accurate or practically suited as clinical tests (Kamath et al 1991), and it appears that no one test, combination of tests, examinations, or observations can accurately predict PNF. Methylglycinoxylidide (MEGX) a dynamic test of liver function that determines lignocaine clearance, has been used to assess the quality of the donor graft with encouraging results (Adam et al 1991, Buckel et al 1993). This test has been championed as a measure of

both liver perfusion and function and thus a predictor of early graft viability (Schroeder et al 1991). This examination can be performed to assess function before retrieval of the graft (D'Alessandro et al 1991). Its place in assessing outcome of graft function is however still uncertain. Rending et al (1993) have failed to show a correlation between donor monoethylglycinexylidide levels and the occurrence of PNF. Adams et al (1993) however, have found that grafts with high MEGX values (>90ng/ml) were associated with good function. The authors however highlighted the limited predictive power of the test and suggested that grafts with a score less than 90ng/ml could still be used.

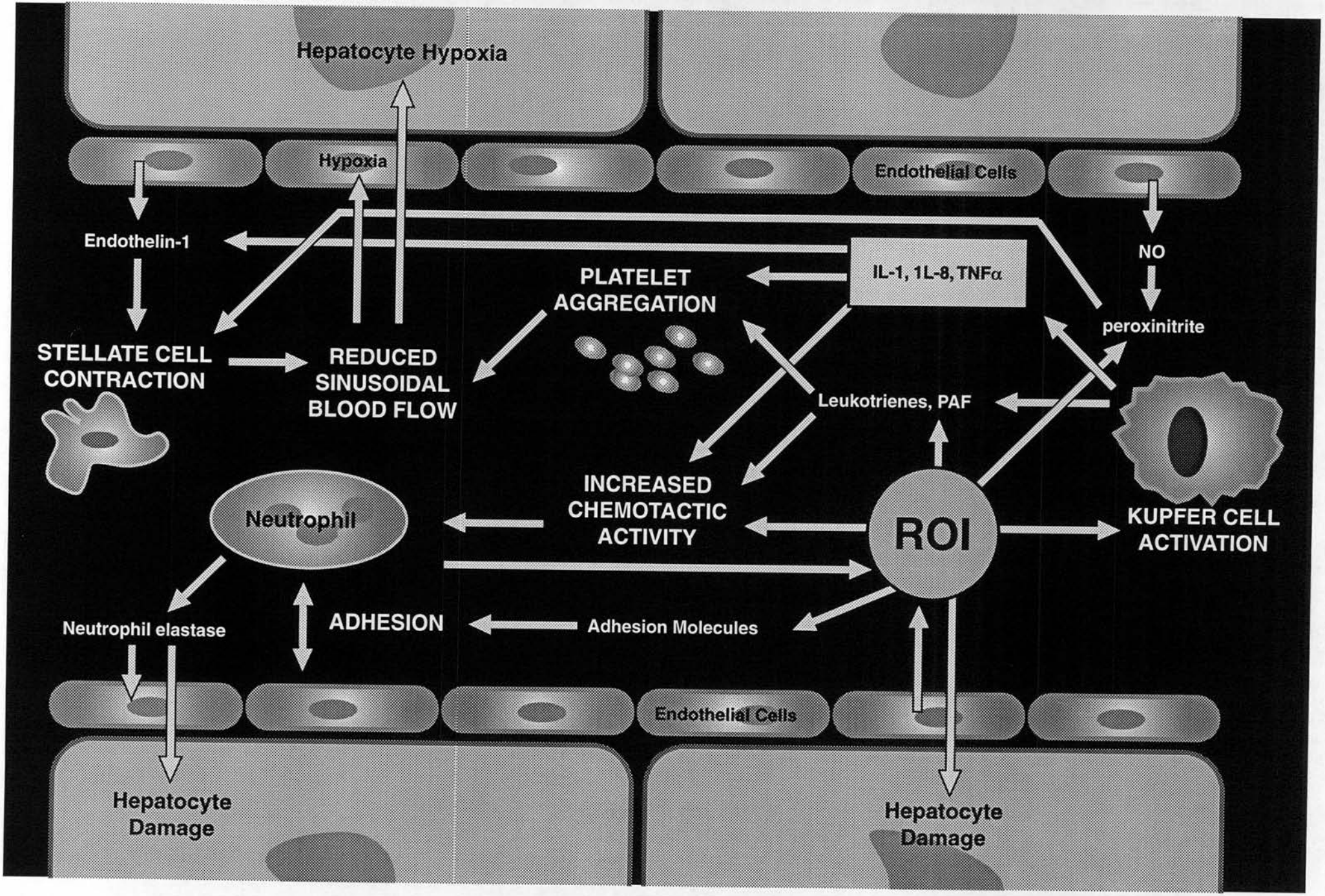
This thesis has shown that an ICG clearance <200 ml/min measured within 24 hrs following liver transplantation predicted graft dysfunction due to reperfusion injury with high specificity and sensitivity. ICG clearance is a safe, relatively cheap and easy to perform test with immediate results. NIMRS technology can simplify the measurement of ICG clearance and may increase further its usefulness further by permitting frequent bedside monitoring of the clearance. Future studies should be directed towards exploring the potential use of ICG clearance in determining suitability of liver graft before retrieval at the donor bedside. Should the test prove to be successful for such a purpose, then the potential for improving the outcome of transplantation is likely to be significant. With the shortage in organ required for transplantation, the test may also help in minimising the

number of grafts turned down because of potential poor quality as judged by our current inadequate measures.

**Reperfusion Injury: Prevention & Thoughts For Future Studies:**

The recent studies and the findings reported in this thesis suggest an importance of the humural changes during the reperfusion phase that could influence the immediate post-operative outcome and development of primary graft dysfunction. The findings of our studies have shown an important role for both neutrophils and ROIs in PGD as a consequence of ischaemia-reperfusion. It is likely that the interaction of neutrophils and ROIs at different stages occur for augmented damaging effects to tissues but remains unclear which one has the more important role. The answer to this question is essential in order to develop a specific preventative and/or therapeutic measures to minimize this injury. The pattern of early increase in ROIs followed by a more sustained increase in NE points favourably towards an earlier activation of the hypoxanthine-xanthine oxidase pathway, which in turn contribute to recruitment and activation of neutrophils. As detailed in the first chapter of this thesis, ROIs enhance the release of mediators such as, PAF and $LTB_4$, and upregulate the expression of specific ligand/adhesion molecules on the luminal surfaces of the sinusoidal endothelial cells. These molecules interact with complimentary ligands on the surfaces of circulating neutrophils resulting in endothelial cell/neutrophil adhesion leading to neutrophil activation and

proteolytic enzymes release which results in further ROI generation (figure 6-1).


Hepatocyte Hypoxia
Hypoxia
Endothelial Cells
Endothelin-1
NO
IL-1, 1L-8, TNFα
PLATELET AGGREGATION
peroxinitrite
STELLATE CELL CONTRACTION
REDUCED SINUSOIDAL BLOOD FLOW
Leukotrienes, PAF
INCREASED CHEMOTACTIC ACTIVITY
Neutrophil
ROI
KUPFER CELL ACTIVATION
Neutrophil elastase
ADHESION
Adhesion Molecules
Endothelial Cells
Hepatocyte Damage
Hepatocyte Damage

At present, no effective preventative treatment exists for reperfusion injury following organ transplantation. Prostaglandins have been studied in relation to reperfusion injury with variable results of success. Greig et al (1989) demonstrated an improvement in graft and patient survival following infusion of $PGE_1$ in patients with evidence of PNF. In a double blind placebo controlled study by Henley et al (1995), $PGE_1$ was administrated intravenously to OLT recipients for 21 days starting intra-operatively. No difference was found between the two groups in relation to the incidence of acute cellular rejection or PNF. They however, found a reduction in the duration of hospitalization, and the need of renal support in the $PGE_1$ group. Antioxidants such as allopurinol, superoxide dismutase, N-acetylcysteine, and s-Adenosylmethionine have shown promising results in preventing reperfusion injury in animal models (Koo et al 1992, Kobyashi et al 1991, Vivot et al 1993, Dunne et al 1994), and warrant further study of their role in human liver transplantation. The selection of an antioxident must take into account both efficacy and safety. Allopurinol for instance acts via the hypoxanthine-xanthine oxidase pathway, without affecting the degree of ROI production by the activated neutrophil which is an equally important mechanism. N-acetylcysteine on the other hand acts by replenishing glutathione stores and is likely to be more effective, but has the disadvantage of reducing tissue extraction of oxygen in cirrhotic patients (Jones et al 1994). A recent study has shown a possible role of s-

Adenosylmethionine (SAMe) if given perioperatively in attenuating reperfusion injury (Dunne et al 1994). SAMe is an endogenous methyl group donor, a precursor of adenosine and GSH. We have discussed earlier in this review the evidence for the protective role of adenosine against graft loss in the studies that followed selective elimination of individual components of either UW or Carolina rinse solution. The effects of adenosine are probably more significant at the end of the ischaemic phase during rewarming of the graft when ATP stores fall rapidly, and following reperfusion through inhibition of ROI release from the activated neutrophils (Bruce, 1985). Such effects would augment the antioxidant capacity of GSH. A further advantage of SAMe is its safety profile in patients with chronic liver diseases as determined by the well documented pharmacokinitec studies (Kaye et al 1990, Manzillo et al 1992).

Endothelins, with their vasoconstrictor effects might have a role to play in reperfusion injury and graft dysfunction, Okumara et al (1991) and more recently Nakamura et al (1995) evidence for no-reflow and sinusoidal circulatory stasis with an increase of endothelin levels. Inhibition of of endothelin-1 release by mono-clonal antibodies in animal models in the latter study lead to improved graft viability. In our study, in human OLT endothelins remained similarly elevated post-reperfusion when compared to the pre-transplant levels which in a sense limit the significance of their role in reperfusion injury. As the purpose of our study was originally to

assess the changes in vasomotor mediators (cGMP and endothelins) in the context of peri-transplant haemodynamic alterations, it would be difficult to extrapolate our findings on reperfusion injury. It however, opens the debate again about the role of endothelins in ischaemia-reperfusion, and it suggests a need for a more detailed study with a larger number of transplant patients to address this question.

**Reperfusion Syndrome:**

This thesis confirmed the findings of previous studies of reperfusion syndrome with the hallmark of haemodynamic instabilites that could have fatal consequences. The thesis has shown that depletion of cGMP during reperfusion was associated with adverse haemodynamic instabilities particularly in the pulmonary circulation. The current knoweldge suggests that the reduction in cGMP activity is probably not a primary phenomenon but rather secondary to the effects of humural changes particularly the ROIs released following ischaemia-reperfusion. Studies assessing the impact of antioxidant therapy on the haemodynamic changes and cGMP activity during transplantation would be of value in elucidating the extent of this relationship.

Attempts to replenish cGMP directly by adding the cGMP analog 8-(4-chlorophenylthio)-guanosine-3',5'-cGMP to the preservation fluid in an experimental rat model of lung transplantation lead to significant reduction

of PVR and an increase in pulmonary artery blood flow after reperfusion (Pinsky et al 1994). Also, NO inhalation was used successfully during OLT to control further rise in PAP in patients with hepatopulmonary syndrome. Such reports supports the findings in this thesis and raises the interest for further studies to explore the full potential of preventing cGMP depletion using measures such as those discussed above.

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# APPENDIX

5

REVIEW ARTICLE

# Primary Graft Dysfunction After Liver Transplantation: From Pathogenesis to Prevention

*Khalid I. Bzeizi, Rajiv Jalan, John N. Plevris, and Peter C. Hayes*

After orthotopic liver transplantation (OLT), some degree of clinical and biochemical dysfunction almost invariably occurs, the severity of which correlates with the degree of hepatic injury. This early injury, known as primary graft dysfunction (PGD), varies from minor insignificant abnormality to primary nonfunction (PNF). The incidence of PNF varies widely depending on the criteria used for its definition.[1] The reported incidence in recent studies has been up to 7%.[2-4] If the refined definition of PNF as death or retransplantation within 2 weeks of OLT[5] is adopted, it would then become apparent that this complication is underreported.

Patients with PNF develop encephalopathy, hemodynamic instability, and produce scanty, pale bile. PNF is associated with increasing glucose requirement, coagulopathy, markedly raised plasma transaminases, metabolic acidosis, and renal failure.[6,7] Patients with PGD have increased morbidity, prolonged intensive care,[1] and an increased incidence of subsequent graft rejection.[8]

Histologically, the appearance of PGD ranges from mild cellular infiltration to hepatocyte ballooning with focal or zonal necrosis, along with microvesicular steatosis.[9,10] The ultrastructural findings are classically seen in the sinusoidal lining cells (SLC) and include vacuolisation of endothelial cells, mitochondrialysis, and interruption of plasma membrane resulting in poor rounding of cells (Fig. 1). Inflammatory cells often cluster in areas of microarchitectural distortion and denudation.[11,12]

Considerable advances have been made in our understanding of the pathogenesis of graft dysfunction following OLT. In this article we will review the current literature concerning causes of graft dysfunction and discuss the preventative and therapeutic strategies under consideration.

## Pathogenesis

PGD is likely to be the product of a number of different elements that arise before, during, and after transplantation. These include

- Donor factors
- Factors associated with organ preservation
- Reperfusion

The following section deals with the relative importance of these factors in the pathogenesis of PGD.

### *Donor Factors*

Potential donors with a known history of alcohol or drug abuse, previous liver disease, abnormal liver functions, or a positive viral hepatitis screen are usually excluded. The macroscopic appearance of the liver at the time of retrieval may be a helpful, albeit late, guide in determining graft suitability. It is, however, not uncommon for suboptimal grafts to be transplanted because of the urgency of the recipient's clinical condition and limitations of the donor screening methods.

Fatty infiltration of the donor liver has been suggested as a risk factor for graft dysfunction, and this is frequently related to alcohol abuse.[5,13] Grafts from malnourished, obese, or older donors may also show fatty infiltration. The severity of fatty infiltration is classified according to the percentage of steatotic hepatocytes as mild (30%), moderate (30%-60%), or severe (>60%). Adam et al[14] found no difference in the 1-month survival of grafts with mild to moderate steatosis when compared with nonsteatotic organs. These findings were not supported by Ploeg et al,[15] who reported that moderate steatosis was an independent risk factor for initial poor graft function. Although debate regarding the use of grafts with moderate steatosis continues, severe fatty infiltration should be considered an absolute risk factor for graft failure, and such grafts should be excluded.

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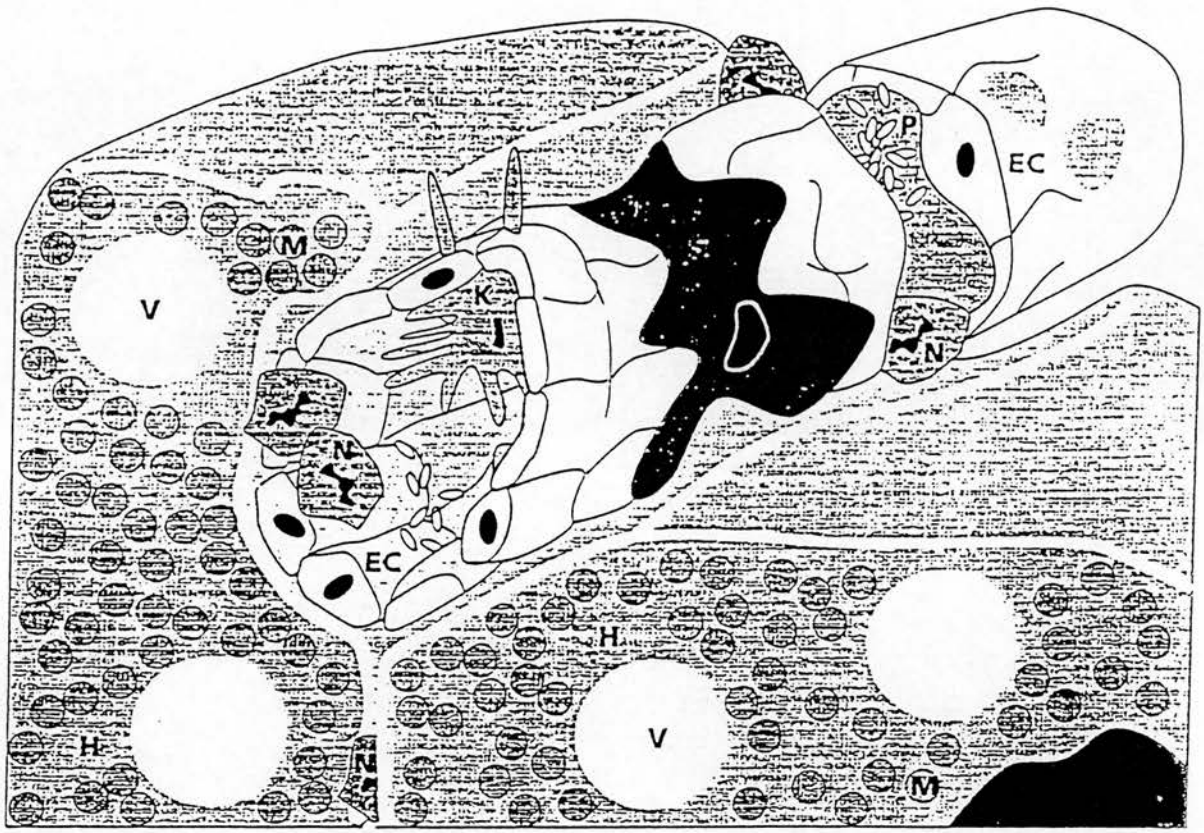


**Figure 1. Schematic representation of the ultrastructural changes of the hepatocytes and sinusoids in liver transplants with poor graft function. Endothelial cells (EC) showing poor rounding with interruption of plasma membranes. Hepatocytes (H), demonstrating vacuolization (V) and steatosis. Mitochondria (M) appear vaculated and enlarged. Rolling and transmigration of activated neutrophils (N). Kupffer cell (K). Aggregated platelets (P) within the sinusoid. Stellate cell (S) shown in a contracted state.**

The mechanism by which fatty infiltration causes graft failure is unclear, although sinusoidal irregularity and narrowing as a result of compression with fat-laden hepatocytes has been suggested as a possible cause.[16] Other studies have implicated enhanced Kupffer cell activity in grafts demonstrating fat infiltration.[17,18]

The effect of donor nutritional status on subsequent allograft function remains unclear. Prolonged fasting has been shown to result in severe hepatocyte deglycogenation, which has been suggested to have a deleterious effect on graft viability.[19,20] Sankary et al.[21] on the other hand, reported that grafts from fasting donors have improved function and suggested that the beneficial effect of fasting was due to reduced Kupffer cell activity.

Donor age has been reported to influence graft viability; however, studies assessing the role of donor age on graft function have shown variable results. While Ploeg et al[15] identified age as a risk factor for graft failure, this was not confirmed in a larger study by Adam et al.[22] Hoofnagle et al[23] assessed the impact of donor age in a cohort of 772 liver transplants. Twenty-five percent of the donors were aged 50 years or above. Intraoperatively, there was poor bile production by grafts of the older donors as compared to younger ones. Graft survival was significantly less for recipients of older donor livers at 3 months (82% v. 91%). Of importance is that the grafts of older donors, which are judged to be good by the harvesting surgeon, were not associated with a decrease in patient or graft survival after OLT. Grafts from elderly donors often demonstrate steatosis, which may be the major factor in determining quality, rather than age alone.

### *Organ Preservation*

Reduction in metabolic rate and energy requirements by induction of hypothermia is the primary principle behind prolonged organ preservation;

however, some degree of metabolic activity occurs even in tissues preserved at 1°C, causing depletion of high energy phosphates in the donor organs. Tissues preserved in the University of Wisconsin (UW) solution at 4°C for 48 hours demonstrate a reduction in the concentration of adenosine triphosphate (ATP) by 70%.[24] This degree of reduction in the concentration of ATP would occur over 2 hours if the tissue is stored at room temperature. Further, metabolic changes that occur as a consequence of hypothermia include acidosis due to the accumulation of lactate from anaerobic glycolysis. Cellular injury is further exacerbated by inhibition of $Na^+/K^+$ ATPase,[25] which results in the accumulation of intracellular sodium. This results in water influx, which causes cell swelling and cell death.

Depletion of ATP during hypoxia damages the $Ca^{++}$ pump, which leads to an increase in the cytosolic free $Ca^{++}$. This increase has been postulated to initiate a cascade of cellular events that culminate in cell death. Specifically, $Ca^{++}$ influx into mitochondria leads to uncoupling of oxidative phosphorylation with further depletion of ATP. $Ca^{++}$ also activates proteases, and this in turn leads to conversion of xanthine dehydrogenase into XO.[26] Upon reperfusion and oxygenation, XO acts on hypoxanthine (which accumulates during hypoxia from the breakdown of ATP) to produce reactive oxygen intermediates (ROI), such as superoxide, and the hydroxyl group (Fig. 1).

Prolonged cold ischemia (beyond 16 hr) has been shown in a rat model to be associated with sinusoidal endothelial cell damage and activation of Kupffer cells. These events result in microcirculatory changes, which are characterized by reduced and uneven blood flow and increased leukocyte and platelet adhesion. Reduction in Kupffer cell activity with nisodipine or pentoxiphylline improves graft survival.[27]

### *Reperfusion Injury*

Tissue damage that follows ischemia-reperfusion has been the focus of considerable research over the last decade. Majno et al[28] described the no-reflow phenomenon in the cerebral circulation in which reduction or complete cessation of blood flow was observed upon reperfusion of ischemic tissue. Koo et al[29] studied changes in blood flow in the liver sinusoids with in-vivo fluorescence microscopy in an ischemia-reperfusion model and showed complete stasis in the majority of sinusoids 30 minutes after reperfusion.

Ultrastructural examination of the SLC after ischemia-reperfusion has demonstrated rounding of cells, focal denudation, adhesion of inflammatory cells, and platelet aggregation. In this same study the extent of reperfusion injury was reduced with the ROI scavenger, allopurinol.

Various inflammatory mediators such as adhesion molecules and leukotrienes have been implicated in the pathogenesis of reperfusion injury. Vasomotor mediators such as nitric oxide (NO) and endothelin have also been shown to contribute to reperfusion injury. In the following section, the relative role of these agents and their interrelationship in the context of reperfusion injury is discussed.

#### Reactive Oxygen Intermediates

ROI are characterized by the presence of one or more unpaired electrons in their outer orbits, which make them highly reactive and potentially toxic. On reperfusion, the enzyme xanthine dehydrogenase is converted to XO within endothelial and Kupffer cells.[30] Hypoxanthine, a metabolite of ATP that accumulates during hypoxia, reacts with oxygen in the presence of XO to produce ROI such as superoxide and the highly reactive hydroxyl group. This is formed by the Haber-Weiss reaction and requires transitional metal ions as a catalyst[31] (Fig. 1).

ROI mediates cellular injury by a number of mechanisms. First, it interacts nonspecifically and indiscriminately with adjacent molecules such as lipids, proteins, and nucleic acids.[31] Second, it increases signal transduction, which results in neutrophil chemotaxis and activation.[32] Third, it reacts with NO to produce the highly toxic peroxynitrite radical.[33]

Studies in both humans and in animal models have shown increased ROI production after reperfusion.[34-36] Antioxidants such as allopurinol and superoxide dismutase[29,37] have been shown to significantly reduce reperfusion injury. Infusion of allopurinol perioperatively in rats undergoing liver transplantation with the grafts preserved in UW solution was associated with a reduced production of ROI and improved graft function.[38] We have also shown that ROI production continues for 24 hours after OLT and that the degree of free radical production correlates with indices of graft function.[39,40] This suggests that ROI is an important mediator of reperfusion injury and its production occurs not only during the reperfusion event but continues for some time after reperfusion. The

prime candidates for this later production of ROI are inflammatory cells, particularly neutrophils, which are known to have a significant capability to generate ROI upon activation.

### Inflammatory Cells

The role of neutrophils in the cellular injury of ischemia-reperfusion is supported by histological studies, which show an accumulation of neutrophils in the injured tissue.[41] This accumulation is reduced by antineutrophil serum or monoclonal antibodies.[42,43] Walden et al[44] demonstrated an improvement in the contractile function of skeletal muscles subjected to ischemia-reperfusion by pretreatment with vinblastin to reduce the neutrophil population.

The process of cellular infiltration starts with adhesion of the neutrophil to the sinusoidal cell. The nature and magnitude of these adhesive interactions depend on the expression of various mediators such as the adhesion receptor/ligand molecules, leukotriene $B_4$ ($LTB_4$), platelet activating factor (PAF), and cytokines such as tumor necrosis factor (TNF)α, interleukin (IL)-1, and IL-2.[45-49] Granule membrane protein (GMP140) and endothelial leukocyte adhesion molecule (ELAM-1), members of the selectin family, increase significantly in the early phase of reperfusion during liver transplantation. This is followed a few hours later by expression of other adhesion molecules such as the vascular cell adhesion molecule (VCAM-1) and the intercellular adhesion molecule (ICAM-1).[45]

The cellular injury produced by neutrophils is due to release of proteolytic enzymes (elastases, proteinases, and collagenases) from their granules and production of ROI through activation of nicotinamide adenine dionucleotide phosphate (NADPH) oxidase. Neutrophil elastase can degrade almost all the components of the extracellular matrix, attack intact cells, and inactivate various proteins such as immunoglobulins, complement, and clotting factors.[50]

Neutrophil elastase and ROI interact at number of levels. $Alpha_1$-antitrypsin, a natural inhibitor of elastase, is inactivated by ROI through posttranslational oxidation of methionine.[51] Neutrophil elastase, on the other hand, enhances further production of ROI from endothelial cells by catalyzing the conversion of xanthine dehydrogenase to XO, an action that can be inhibited by elastase inhibitors, $alpha_1$-antitrypsin, and elastinal.[52] Such interactions among these mediators potentiates their damaging influence on the tissues affected.

ROI promotes adherence of leukocytes to the microvascular endothelium, and this effect is thought to be mediated by induction of adhesion molecules such as GMP140 and by activation of phospholipase $A_2$ ($PLA_2$), which in turn increases $LTB_4$ synthesis.[53,54] The interaction between ROI and adhesion molecules in the process of leukocyte-endothelium adhesion has been demonstrated in a feline intestinal muscle model. Upon superfusion with hypoxanthine-XO there was a rapid increase in the number of rolling leukocytes, which is an early and essential event prior to adhesion. The use of monoclonal antibodies to P-selectin before superoxide exposure inhibited the recruitment of rolling leukocytes.[55]

### Kupffer Cells

The role of Kupffer cells in ischemia-reperfusion has been demonstrated in a number of studies. In a rat model, when hypoxia was selectively induced in pericentral regions of the liver, electron microscopic analysis of Kupffer cells revealed numerous lamellapodia and pseudopodia. These changes indicate activation of Kupffer cells. This activation can be prevented by pretreatment with methyl-palmitate.[56] Following activation, Kupffer cells release a number of inflammatory mediators such as IL-1 and IL-8, which attract neutrophils and also produce ROI.[57-59] Activated Kupffer cells also release TNFα, which has been shown to induce production of endothelin (ET)-1 by endothelial cells. Inhibition of Kupffer cell activation with gadolinium chloride or adenosine-2 receptor agonists such as nisoldipine and prostaglandin $E_1$ has been shown to prevent microcirculatory disturbances after reperfusion.[60,61]

### Stellate Cells

The hepatic stellate or Ito cell is located in the space of Disse and resembles the pericytes localized around capillaries. Its role in the regulation of sinusoidal liver blood flow is being increasingly recognized. Stellate cells contract in response to a variety of vasoactive substances, including ET-1 and substance P.[62,63] They are also activated by free radicals.[64] Several vasoactive substances are released during reperfusion injury that can potentially stimulate stellate cells causing sinusoidal contraction with subsequent stasis, hypoxia, and further release of inflammatory mediators to complete the vicious circle leading to graft dysfunction.

### Nitric Oxide

NO is another important modulator of leukocyte adhesion that has been implicated in the pathogenesis of reperfusion injury. NO is enzymatically synthesized from L-arginine by NO synthase. Its predominant function is relaxation of vascular smooth muscle and inhibition of platelet aggregation.[65] It also inhibits neutrophil-endothelium adhesion by down-regulating the expression of the ligand P-selectin[66] and decreases ROI production by inhibiting neutrophilic NADPH oxidase.

NO is the only known biological molecule that can be produced in high enough concentration to compete with ROI for superoxide dismutase. The interaction results in formation of peroxynitrite, a radical in its own right that, unlike NO, induces vasoconstriction.[67] This may contribute to sinusoidal stagnation and the no-reflow phenomenon seen in reperfusion injury.

NO causes vasodilatation through its secondary mediator cyclic guanosine monophosphate (cGMP), which is produced from the activated guanylate cyclase in endothelial cells.[68] ROI has a direct inhibitory effect on guanylate cyclase, which results in a reduction in intracellular cGMP levels,[69] which is probably a further contributing factor involved in microcirculatory stagnation and reperfusion injury.

### Endothelins

Endothelins are a family of vasoactive peptides, of which three forms have been identified (ET-1, ET-2, and ET-3). All isoforms contain 21 amino acids but differ in the two and five amino-acid residues. Endothelial cells appear to produce ET-1 exclusively, which is considered the most important biological vasoconstrictor, acting on both venous and arterial smooth muscle cells.[70]

Plasma levels of endothelins are increased markedly in the reperfusion phase after cardiac ischemia in rats, as well as in patients undergoing cardiac transplantation.[71,72] Administration of ET-1 to rats results in significant hepatic sinusoidal constriction and a reduction of blood flow in a dose-dependent manner.[73] ET-1 could therefore mediate the no-reflow phenomenon, which is the hallmark of reperfusion injury.

### Leukotrienes

Leukotrienes are important inflammatory mediators that are metabolites of arachidonic acid produced by the 5-lipoxygenase pathway, primarily in Kupffer cells. The concentration of $LTB_4$, a potent chemoattractant, increases markedly following ischemia-reperfusion.[74,75] The leukotriene biosynthesis inhibitor MK-886 has been shown to prevent postischemic leukotriene accumulation and the

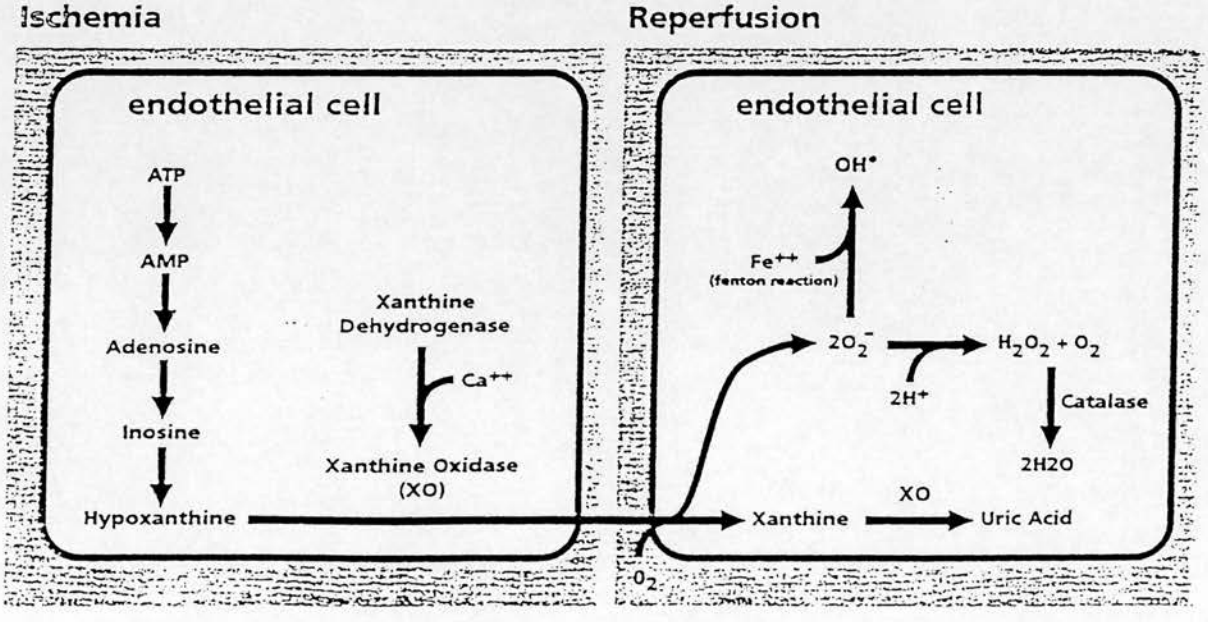


Figure 2. Diagrammatic representation of classical pathway of ROI generation during ischemia/reperfusion. (OH, hydroxyl radical; $H_2O_2$, hydrogen peroxide.)

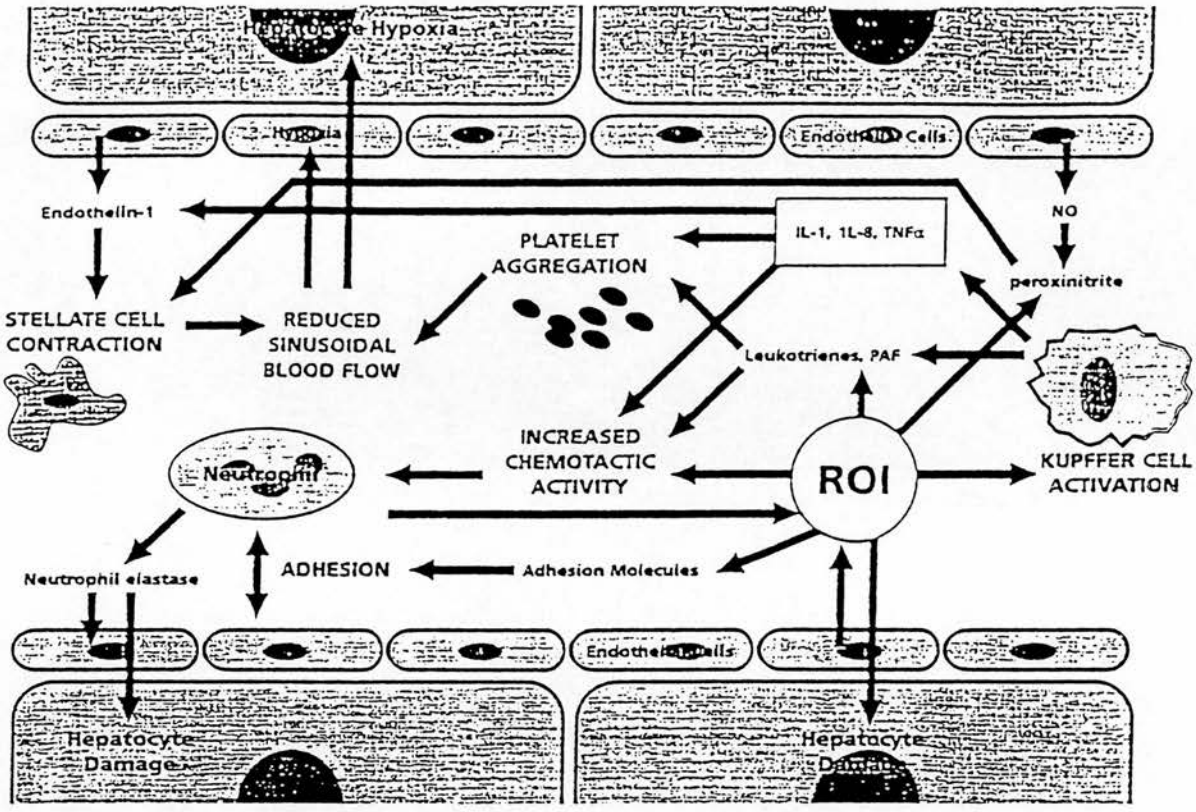


**Figure 3. Schematic representation of the cellular and intravascular events in relation to ischemia/reperfusion following liver transplantation.**

microcirculatory changes of ischemia-reperfusion injury.[54]

The relationship between leukotrienes and ROI has been studied, and it seems that ROI, by increasing the intracellular free calcium, activates plasma membrane $PLA_2$, which in turn enhances leukotriene production by converting cell membrane phospholipids into arachidonic acid.[76]

### Platelet Activating Factor

PAF is an autacoid phospholipid with vasoactive and proinflammatory activity that has been studied in relation to reperfusion injury. Its main functions are to induce platelet aggregation and prime inflammatory cells to release various mediators such as proteases and cytokines.[77]

ROI enhances the release of PAF from endothelial cells, leukocytes, and platelets by increasing the permeability of endothelial cells and platelets to calcium flux, which would activate the $PLA_2$.[78] Studies of PAF have shown increased levels after reperfusion, but the use of the specific inhibitor WEB-2170 in the hepatic ischemia-reperfusion rat model did not protect against injury.[79] Its role in the pathogenesis of reperfusion injury remains unclear.

## Prevention

### *Graft Selection*

Selection criteria for liver grafts are somewhat arbitrary, and more specific and objective measures are required. The limited time available before retrieval undoubtedly imposes a significant constraint on the ability to determine suitability of the graft. Liver function tests such as serum transaminases and measurement of prothrombin time are helpful but have limited sensitivity and specificity. Frozen section liver biopsy, for instance, should be considered whenever doubt about graft quality exists.

Methylglycinoxylidide (MEGX), a dynamic test of liver function that determines lignocaine clearance, has been used to assess the quality of the donor graft with encouraging results.[22,80] It is easy

to perform, relatively cheap, and provides rapid results. In this test, lignocaine clearance is determined by the activity of the cytochrome P-450 system, and, therefore, concomitant drug use can affect the sensitivity of this test. Adams et al[81] have found that grafts with high MEGX values (>90 ng/mL) were associated with good function. The authors, however, highlighted the limited predictive power of the test and suggested that grafts with a score less than 90 ng/mL could still be used.

Indocyanine green (ICG) clearance is another dynamic test of liver function with potential value in determining suitability of grafts for retrieval. We have shown that ICG predicted subsequent graft function within 24 hours after transplantation.[82] ICG values below 200 mg/mL indicate poor graft function in our experience.

Table 1. Contents of UW Solution

| Contents | Concentration |
|---|---|
| Raffinose | 30 mmol/L |
| K-lactobionate | 100 mmol/L |
| Hydroxyethyl starch | 50 g/L |
| Adenosine | 5 mmol/L |
| Allopurinol | 1 mmol/L |
| Glutathione | 3 mmol/L |
| Insulin | 40 U/mL |
| $MgSO_4$ | 5 mmol/L |
| $KH_2PO_4$ | 25 mmol/L |
| Penicillin | 200 U/mL |
| Dexamethasone | 16 mg/L |
| Osmolarity | 310-330 |
| pH | 7.4 |

## *Organ Preservation*

Advances in methods of organ preservation have contributed significantly to the progress and widespread application of liver transplantation in the management of acute and chronic liver disease. The UW solution has an established place in organ preservation and has made it possible to preserve grafts for a period of up to 24 hours before transplantation. This allows retrieval and transport of grafts over considerable distances, thereby greatly increasing the number of grafts available for transplantation. Surgeons can now choose convenient times for the operation, and it is becoming possible for the same surgeon to undertake both the retrieval and the transplant operation.

Despite these advances, however, the reported incidence of primary graft dysfunction has not changed significantly. This emphasizes the need for further improvements in preservation techniques.

The composition of the UW solution is complex (Table I), and the value of some of its components has not been clearly defined. Adenosine, a precursor for ATP synthesis, is probably important during warm ischemia when ATP breakdown is accelerated.[83] Adenosine is also known to inhibit production of ROI from activated neutrophils by interacting with the adenosine A-2 receptors on their membranes.[84] Allopurinol and glutathione are included as scavengers of ROI, whereas lactobionate and raffinose, which are large organic molecules (molecular weight of 358 and 594, respectively), provide osmotic support and prevent cell swelling by interfering with membrane permeation.[85] Recently, it has been found that elimination of allopurinol, phosphate buffer, and $MgSO_4$ does not affect graft survival after 9 hours of preservation. Graft survival, on the other hand, improved upon elimination of insulin, which raises questions about its inclusion in UW solution.[86]

Addition of agents such as calcium channel blockers, tryptophan, chlorpromazine, and glycine have shown variable results, with the last being most promising.[86-89] It probably acts by inhibiting phospholipase, blocking calcium channels, and stabilizing cell membranes. Lazaroid U74006F (21-aminosteroid) is a potent inhibitor of iron-dependent peroxidation, which has been shown to reduce superoxide anion activity. When added to UW solution, there was a significant reduction in transaminases after OLT.[90]

Preliminary studies suggest an advantage in adding the immunosuppressive drug tacrolimus (FK506) to UW solution. The mechanism of its cytoprotective effects is probably nonimmunological. Its effect is thought to be through recovery of tissue ATP, which minimizes cellular injury during warm ischemia.[91]

Misoprostil, a prostaglandin $E_1$ analogue, demonstrated a protective effect against hepatocellular injury in a rat model of ischemia-reperfusion. The mechanism of action is thought to be mediated through inhibition of endothelial cell–neutrophil adherence and production of ROI.[92]

Limiting the duration of ischemia remains an important priority because the incidence of graft dysfunction increases rapidly with cold storage beyond 12 hours.[93]

Flushing solutions are used before reperfusion of the graft to clear $K^+$, which is present in high concentrations in UW solution. Carolina rinse solution (Table 2) improves graft survival after prolonged ischemia in UW solution. The efficacy of this solution seems to depend largely upon the presence of glutathione, allopurinol, and adenosine. Selective elimination of these components, particularly adenosine, resulted in reduced graft survival.[86]

### *Reperfusion*

Despite the large number of studies demonstrating evidence of reperfusion injury after organ transplantation, there is at present no effective preventive treatment.

Prostaglandins have been studied in relation to reperfusion injury with variable degrees of success. Greig et al[94] demonstrated an improvement in graft and patient survival following infusion of prostaglandin $E_1$ in patients with evidence of PNF. In a double-blind, placebo-controlled study, Henley et al.[95] administered prostaglandin $E_1$ intravenously to OLT recipients for 21 days, starting intraoperatively. No difference was found between the two groups in relation to the incidence of PNF or acute cellular rejection. The investigators did find, however, a significant reduction in the duration of hospitalization and the need for renal support in the prostaglandin $E_1$ group.

Antioxidants such as allopurinol, superoxide dismutase, *N*-acetylcysteine, and *S*-adenosylmethionine (SAMe) have shown promising results in preventing reperfusion injury in animal models[29,96-98] and warrant further study of their role in human liver transplantation. The selection of an antioxidant must take into account both efficacy and safety. Allopurinol, for instance, acts through the hypoxanthine-XO pathway without affecting the degree of ROI production by the activated neutrophil, which is an equally important mechanism. *N*-acetylcysteine, on the other hand, acts by replenishing glutathione stores and is likely to be more effective, but it has the disadvantage of reducing tissue extraction of oxygen in cirrhotic patients.[99] A recent study has shown a possible role for SAMe, if given perioperatively, in attenuating reperfusion injury.[98] SAMe is an endogenous methyl group donor, a precursor of adenosine and glutathione. We have discussed in this review the evidence for the protective role of adenosine against graft loss in the studies that followed selective elimination of individual components of either UW or Carolina rinse solution. The effects of adenosine are probably more significant at the end of the ischemic phase, during rewarming of the graft, when ATP stores fall rapidly, and after reperfusion through inhibition of ROI release from the activated neutrophils.[34] Such effects would augment the antioxidant capacity of glutathione. A further advantage of SAMe is its safety profile in patients with chronic liver diseases as determined by the well-documented pharmacokinetic studies.[91,92]

**Table 2.** Contents of Carolina Rinse Solution

| Contents | Concentration |
|---|---|
| Hydroxyethyl starch | 50 g/L |
| $MgSO_4$ | 1.2 mmol/L |
| $KH_2PO_4$ | 1 mmol/L |
| Glutathione | 3 mmol/L |
| Allopurinol | 1 mmol/L |
| Insulin | 100 U/L |
| Adenosine | 0.2 mmol/L |
| Nicardipine | 2 umol/L |
| Desferrioxamine | 1.0 mmol/L |
| Fructose | 10 mmol/L |
| $CaCl_2$ | 1.3 mmol/L |
| KCl | 5 mmol/L |
| MOPS* | 20 mmol/L |
| NaCl | 115 mmol/L |
| Glucose | 10 mmol/L |
| Osmolarity | 290-305 |
| pH | 6.5 |

## Conclusions

PGD is an important complication after liver transplantation, and in the last few years, considerable advances in our understanding of its etiological mechanisms have been made. Undoubtedly, more questions are yet to be answered about the degree of interactions among the various contributing factors in its development. Donor selection criteria, although significantly improved, need to be refined further. More emphasis should be given to assessing the currently available and promising dynamic liver tests in addition to developing other discriminatory measures that can deliver rapid and reliable answers regarding graft suitability.

Preservation and rinse solutions that have been

important in establishing the success of liver transplantation need to be further modified to maximize benefit. Components such as insulin, phosphate, and $MgSO_4$ could probably be eliminated from UW solution without adversely influencing graft viability. On the other hand, addition of substances such as tacrolimus and NAC may improve graft function.

It is evident from this discussion that a large number of mediators are released following reperfusion that are capable, individually or collectively or perhaps in a cascade fashion, of activating Kupffer cells and initiating neutrophil-endothelial cell interactions. The detrimental effects of ROI are mediated by directly damaging tissues and by their capacity to stimulate the release of mediators such as PAF, $LTB_4$, and the selectin family of the adhesion molecules. These are important in the process of chemoattraction, adhesion, and activation of neutrophils (Fig. 3). Strategies to prevent ROI production with pharmacological modalities such as SAMe are likely to be of potential clinical value. Experimental studies have shown evidence of attenuation of reperfusion injury by using monoclonal antibodies to block P-selectin–dependent neutrophil-endothelial interactions. Further work is also required to explore the benefits of measures aiming to inhibit activation of Kupffer cells with agents such as adenosine-2 receptor agonists.

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resolution of menopausal symptoms she stopped the HRT (and tamoxifen) after only 3 months. 12 months later she developed a pleural effusion and probable lung metastases.

Tamoxifen did not seem to compromise the effectiveness of HRT. There is no evidence of rapid disease progression in these patients receiving HRT and tamoxifen. Thus, it may be possible to design hormone replacement that retains the ability to control menopausal symptoms while protecting against breast cancer.[2] Nevertheless, there is an urgent need for randomised trials of HRT, with or without tamoxifen, before such untested treatments become common.

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1 Early Breast Cancer Trialists' Collaborative Group. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy: 133 randomised trials involving 31000 recurrences and 24000 deaths among 75 000 women. *Lancet* 1992; 339: 71–85.
2 Stoll BA. Hormone replacement therapy for women with a past history of breast cancer. *Clin Oncol* 1990; 2: 309–12.

## Mysterious respiratory disease in USA

SIR—Details of the mysterious respiratory illness mentioned by Nelson (June 12, p 1526) among Navajo Indians are at present sparse, but the symptoms are reminiscent of those described early in the course of an "acute nephritis" which occurred with trench warfare in the First World War.[1] Although proteinuria and fluid retention was the norm, many soldiers presented with acute dyspnoea and probably low pressure pulmonary oedema, although death from the acute respiratory phase was rare. The cause was never known but affected soldiers all had close contact with soil—which may explain why the cavalry and officers were rarely affected.

A similar illness was seen during trench warfare in the American Civil War. In data quoted by Langdon Brown,[1] the condition appears to have been more common in summer months, and in June–July, 1862, there were 148 deaths. It has been proposed by several workers[2] that trench nephritis was caused by hantavirus, although the classic descriptions from the First World War do not tally closely with the variants of hantavirus infection that have been described in the past 40 years.

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1 Brown WL. Trench nephritis. *Lancet* 1916; i: 391–95.
2 Fisher-Hoch SP, McCormick JB. Haemorrhagic fever with renal syndrome: a review. *Abstr Hyg Common Dis* 1985; 60: R1–R20.

## QT interval, autonomic neuropathy, and alcoholic liver disease

SIR—The finding by Day and colleagues (June 5, p 1423) that prolonged QT interval in alcoholic liver disease is an independent prognostic indicator and associated with sudden death is of considerable interest. Patients in their study initially had no clinical or echocardiographic evidence of heart disease and they suggest that the QT interval abnormalities may have been early indicators of alcohol-induced myocardial toxicity.

Another possibility is that these changes are related to an autonomic neuropathy, which may alter the length of the QT interval in the electrocardiogram.[1] A prolonged interval is seen in diabetic autonomic neuropathy where it is associated with sudden death,[2] and in chronic alcoholics.[3] 50% of patients with alcohol-related liver disease will have evidence of vagal neuropathy on standard cardiovascular reflex tests.[4] Kempler et al,[5] noted a close correlation between prolonged QT interval and vagal neuropathy in both alcohol-related and non-alcohol-related liver disease. Autonomic neuropathy could have contributed to the QT interval abnormalities and increased mortality noted by Day and colleagues.

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1 Esler M. The autonomic nervous system and cardiac arrhythmias. *Clin Auton Res* 1992; 2: 133–35.
2 Kahn JK, Sisson JC, Vinik AI. QT interval prolongation and sudden cardiac death in diabetic autonomic neuropathy. *J Clin Endocrinol Metab* 1987; 64: 751–54.
3 Duncan G, Johnson RH, Lambie DG, Whiteside EA. Evidence of vagal neuropathy in chronic alcoholics. *Lancet* 1980; ii: 1053–57.
4 Hendrickse MT, Triger DR. Peripheral and cardiovascular autonomic impairment in chronic liver disease: prevalence and relation to hepatic function. *J Hepatol* 1992; 16: 177–83.
5 Kempler P, Varadi A, Szalay F. Autonomic neuropathy and prolongation of the QT interval in liver disease. *Lancet* 1992; 340: 318.

## Free radical production following vascular reperfusion

SIR—Grech and colleagues (April 17, p 990) show evidence of free radical production in a spin-trapping method after coronary recanalisation. With an indirect method (the ratio between linoleic acid and its phospholipid esterfied conjugated diene isomer by high-performance liquid chromatography[1]), we measured free radicals in a patient who had a liver transplant. We were struck by the similarities between free radical signals that appeared after reperfusion and those noticed by Grech and colleagues (figure). An increase 30 min after reperfusion of the graft was followed by a fall, then a second increase at 12 h.

We believe that the initial rise is related to increased levels of xanthine oxidase that accumulate during the ischaemic phase

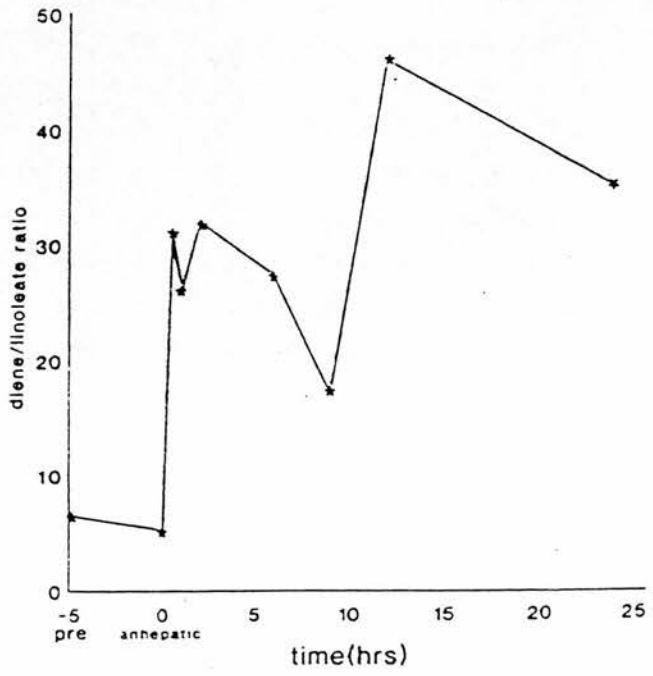


Figure: **Free radical generation after liver transplant**

after organ retrieval. However, this explanation does not account for the second peak. We agree with Grech and colleagues that neutrophil activation may be responsible. A liver biopsy specimen taken from our patient shortly after reperfusion of the graft showed neutrophil infiltration. Since neutrophils are known to have a membrane-associated nicotinamide adenine dinucleotide phosphateoxidase system that generates large numbers of free radicals, this seems a likely source for late free radical generation.

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1 Iversen SA, Garwood P, Dormandy TL. A method for the measurement of diene-conjugated derivative of linoleic acid 18:2(9,11) in serum phospholipid and possible origins. *Ann Clin Biochem* 1985; 22: 137–40.

## Anomalous acetylcholinesterase in CSF without clinical diagnosis of Alzheimer's disease

SIR—Navaratnam et al[1] and Smith et al[2] reported that an anomalous molecular form of acetylcholinesterase (AChE) (also called AChE-AD) could be found in postmortem and antimortem cerebrospinal fluid (CSF) of patients with histopathologically proven Alzheimer's disease (AD) but not in patients without AD. We have found, however, that when more samples were investigated with the same methods, the abnormal AChE is not only associated with AD but also related to other neurological diseases that may cause dementia, although these patients are currently non-demented (Mini-Mental State examination):

| Diagnosis | No of cases | No with AChE-AD band |
|---|---|---|
| Brain cysticercosis | 8 | 3 |
| Myelopathy | 4 | 0 |
| Multiple sclerosis | 4 | 1 |
| Other* | 13 | 1† |
| Total | 29 | 5 |

*Virulent meningitis, Guillain-Barré syndrome, headache, 3 each; intracranial hypertension, 2; tuberculosis meningitis, varicocele, 1 each. † Tuberculosis meningitis.

The positive cases were aged 35–50 (M/F 2/3); the negative cases were aged 15–58 (13/11).

The biochemical and pathological process in AD takes years to develop senile plaques and neurofibrillary tangles (before dementia) and the plaques and tangles are not specific for AD.[3] The AChE is involved early in plaque and tangle formation.[4] Kokmen et al,[5] in an investigation of clinical risk factors for AD, stated: "We are unable to rule out a similar increase in risk with a history of previous meningitis or encephalitis", which indicates a possible relation between AD and our 5 positive cases. Sotelo et al[6] studied 753 patients with brain cysticercosis and found 16% with intellectual deterioration and 3% with disturbances of behaviour. The fact that 3 of our patients had had brain cysticercosis might indicate that an abnormal AChE could be related to the intellectual deterioration in this disease, when a certain number of cysts are located in the regions responsible for memory and cognition. Davis et al[7] reviewed 157 necropsy-proven cases of multiple sclerosis and found 47% of patients with a mental disturbance. In tuberculosis meningitis, Alvarez and McCabe[8] reported 7 cases with abnormal behaviour or altered mental status in a total of 13 patients.

These findings, with the clinical risk study, might form a reasonable explanation for our 5 positive samples, and raise new insights that the CSF AChE-AD may not be specific for AD and may participate in the long biochemical and pathological abnormality of not only AD but also organic dementia. An AChE-AD in the CSF of non-demented patients might mark a "brain at risk", and treatment to clear the abnormal molecular form of AChE should be given with anti-cyst, anti-tuberculosis, and anti-multiple-sclerosis drugs.

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## A walking aid for Parkinsonian patients

SIR—Frequent falls are a common source of embarrassment and injury in patients with parkinsonism. When postural dizziness, fainting, and tripping are excluded, many falls are caused by akinetic freezing—an inability to move the leading foot forward when the trunk starts in motion. Physiologically, there is a failure to select the right agonist muscles in advance of rapid and precise movement; a delay in switching from one movement to another; and a defect in simultaneously activating different parts of the body.[1] Patients find visual proprioceptive cues very useful—eg, aiming at marks or patterns on the floor, trying to kick forward the leading leg, or turning up the toes inside the shoe.

Figure: **Seymour stick**

A patient of mine has partly overcome his trips and freezing of gait by inventing a small horizontal metal bar, attached by a hinge to the bottom of his walking stick, 4 cm above the ground (figure). If he glances at this, he is able to step quickly over it with a good length stride, without tripping or interrupting the rhythmical pattern of normal walking. A friend fashioned this for him at negligible cost.

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# Graft dysfunction following liver transplantation: role of free radicals

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*Background/Aims:* Following orthotopic liver transplantation primary graft non-function occurs in about 10% of patients, and survival depends on early retransplantation. The aetiology has yet to be defined, but reperfusion injury as a result of free radical production has been considered as a possible mechanism. In this study we looked for evidence of free radical generation intraoperatively and assessed the relationship between free radical production and graft function.

*Methods/Results:* Twenty-one patients (M:F 10:11, mean age; 53±3.8 years) who underwent liver transplantation for end-stage liver disease were studied. Free radical activity increased significantly following reperfusion, as shown by: (i) the diene conjugated method, where the percentage molar ratio increased from a baseline of 10.87±0.78% to 24.42±7.8% ($p<0.01$), and (ii) by electron paramagnetic resonance, where a more than a twofold rise in radical concentration was detected ($p<0.05$). The increase in free radical activity detected by the diene conjugated method was significantly higher in patients with poor outcome as compared with those who had uneventful recovery ($p<0.01$).

*Conclusion:* Free radical activity is increased following reperfusion of liver graft during transplantation, and the magnitude of the rise is related to the severity of graft dysfunction.

*Key words:* Free radicals; Liver transplantation; Primary non-function; Reperfusion injury.

Failure of the graft to function following orthotopic liver transplantation (OLT) is common and is recognized in its extreme form as primary non-function (PNF), which occurs in ~10% of patients (1). It is the principal cause of retransplantation in the first 2 weeks post-operatively (2). A less severe form is defined as primary graft dysfunction (PGD), which is associated with increased morbidity, prolonged I.T.U stay and later graft rejection (3).

Features of PNF include marked encephalopathy, haemodynamic instability and poor bile production. Patients demonstrate increasing glucose requirement, coagulopathy, markedly raised plasma transaminases, metabolic acidosis and renal failure.

It is likely that PGD is due to a number of coexisting factors resulting in complications that arise during transplantation, starting with the donor stage through preservation to reperfusion. Koo et al. (4) showed evidence of hepatic microcirculatory stasis which was associated with a significant degree of hepatocellular necrosis following ischaemia-reperfusion in a rat model. Such effects have been shown to be prevented by concomitant administration of superoxide dismutase (SOD), a free radical scavenger.

Reperfusion injury has been the focus of considerable research over the last decade. This has largely concentrated on the release of various inflammatory mediators, such as leukotrienes, adhesion molecules and free radicals, with the latter attracting most attention (4–7). Free radicals are characterized by the presence of one or more unpaired electrons in their atomic orbital. They are highly reactive and potentially toxic. During ischaemia, the enzyme xanthine oxidase (XO) accumulates as a result of calcium influx and activation of proteases. Upon reperfusion, XO acts on hypoxanthine in the presence of $O_2$ and generates radicals such as superoxide and hydroxyl. Antioxidants such as SOD, N-acetylcysteine and

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...in animal models were shown to ameliorate reperfusion injury (8–10).

We aimed in this study to quantitate free radical activity during OLT and to identify the relationship between their magnitude of change and subsequent graft function.

## Materials and Methods

We studied 21 patients (10 males and 11 females with a mean age of 53±3.8 years) who underwent OLT. The indications were primary biliary cirrhosis (9 patients), alcoholic liver disease (6), primary sclerosing cholangitis (3), and cryptogenic cirrhosis (3). The mean donor age was 38±6 years.

Matching of donor liver to the recipient was carried out as per standard protocols. Eleven grafts were retrieved by the same team from our unit, while the rest (10 grafts) were retrieved by a team according to the geographical location of the donor. Generally, each team has followed a standard protocol of retrieval. Grafts with a significant degree of steatosis or of donors with history of drug/alcohol abuse were rejected.

University of Wisconsin (UW) solution was used for preservation, and grafts were flushed with Carolina rinse solution prior to restoration of blood flow. All patients underwent veno-venous bypass during the anhepatic phase; mean duration 70±11 min.

Following OLT, data collected included daily rate of bile production, time to correction of acidosis, serum transaminase, and prothrombin time. For objective assessment of graft function and outcome, we used criteria described previously (11) and these are detailed in Table 1. The scoring system of this criteria is dependent on serum ALT and prothrombin time activity determined at 72 h post-operatively, along with the mean value of bile output over the first 3 days of transplant. In each patient, the score was calculated from the sum of assigned values from each parameter, ranging from 3 to 9. According to these criteria, patients with a total score up to 6 were shown to have good outcome, and a score of 7–9 was associated with poor graft function.

Further data collected included cold ischaemia time, blood transfusion requirements, and acute rejection episodes. The relationship of these parameters with the degree of free radical activity was determined.

TABLE 1

Parameters of scoring system for assessment of early postoperative graft function in liver transplant patients

| Parameter | Assigned value |
|---|---|
| Serum ALT (U/l)[a] | |
| <1000 | 1 |
| 1000–2500 | 2 |
| >2500 | 3 |
| Bile output (ml/day)[b] | |
| >100 | 1 |
| 40–100 | 2 |
| <40 | 3 |
| Prothrombin activity (%)[c] | |
| >60 (spontaneous) | 1 |
| >60 (with fresh-frozen plasma) | 2 |
| <60 (despite fresh-frozen plasma) | 3 |

[a] Highest value in the 72 h after transplant. Normal value is up to 40 U/l.

[b] Mean value during the first 72 h after transplant.

[c] Lowest value in the 72 h after transplant.

*Markers of free radical activity*

The percentage molar ratio (PMR) of diene conjugated linoleic acid (PL-9, 11-LA) to its substrate phospholipase-esterified linoleic acid (PL-9, 12-LA) was used as a determinant of free radical activity. Blood samples were taken from each patient through an indwelling catheter inserted following induction of anaesthesia, with its tip being positioned in the right atrium. Samples were taken following induction of anaesthesia, during the anhepatic phase and following reperfusion at 30 min, 1, 2, 6, 9, 12 and 24 h. Plasma was separated by centrifugation at 3000 G for 15 min and stored at –40°C for later analysis. Plasma concentrations of both (PL-9, 11-LA) and (PL-9, 12-LA) were measured by high performance liquid chromatography after enzymatic hydrolysis, and solid phase separation as described by Iverson et al. (12). The coefficient of variation of the assays was less than 3.5%, and results are expressed as diene conjugated/linoleic acid ratio, to reduce error due to substrate variation.

In a subgroup of 10 patients, we used a spin trapping method as another determinant of free radical activity. This has been described previously (13). In brief, from each patient, blood samples (1 ml each) were withdrawn from the same right atrial catheter after induction of anaesthesia, during the anhepatic phase and following reperfusion at 1, 12 and 24 h. They were immediately mixed with 50 mM solution of the spin trapping agent cc–phenyl N-tert-butyl nitrone (PBN) and stored in liquid nitrogen. The PBN-free radical adduct was extracted by adding 2 ml of toluene to each ml of the mixture followed by centrifugation at 10000 G for 5 min. The toluene extract was separated and stored at –40°C until analysis by electron paramagnetic resonance (EPR) spec-

measured by Bruker ECS106 spectrometer. The microwave power was 20 mW, applied field, 3355±30 gauss and frequency of 9.4 G Hz, using 100 KHz modulation with an amplitude of 0.5 gauss. The tme constant was 0.3 s and the sweep time 84 s. The sample temperature was 220°K.

Free radical concentrations were determined by comparing the double integrals of the EPR signals from the experimental samples with those from known concentrations of a stable free radical, 4-hydroxy–2,2,6,6-tetramethylpiperidine–1-oxyl, in the same solvent and recorded under identical conditions. In those cases where very low signal:noise precluded accurate integration, radical concentrations were estimated by comparison of signal heights of the weaker signals with those of the stronger signals.

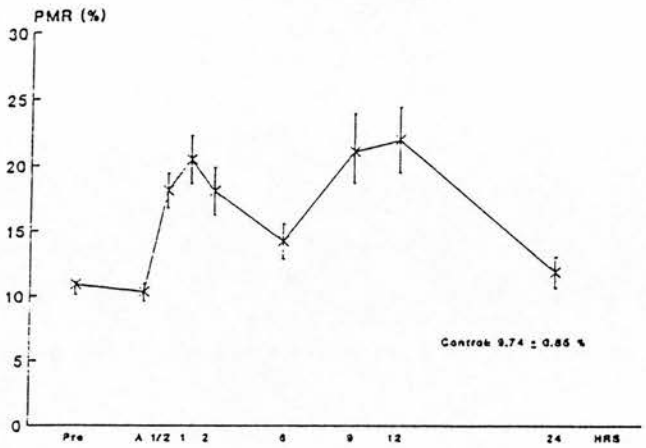


*Fig 1. Peri-operative changes in PMR ratio as a determinant of free radical activity. Pre: pre-transplant, A: During anhepatic phase, and then post reperfusion up to 24 h.*

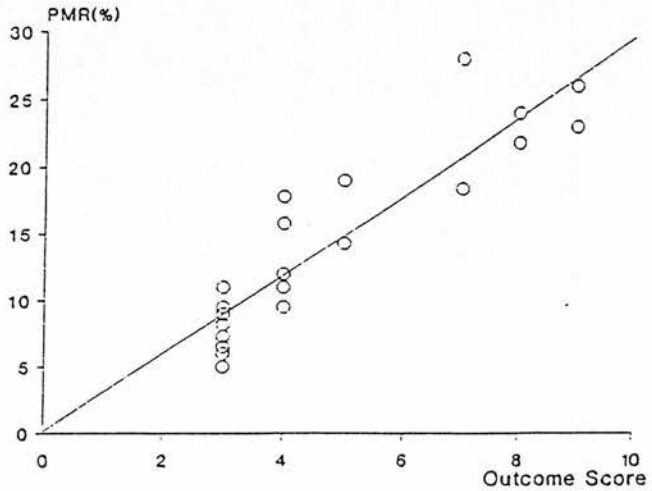


*Fig. 2. Relationship of PMR as a determinant free radical activity to the markers of postoperative graft function as defined by criteria in Table 1.*

*Statistical analysis*

Results were expressed as mean±standard error (SEM). Statistical significance was accepted for $p<0.05$. The difference between time points was calculated using one-way analysis of variance. Correlation between variables was performed by linear regression analysis using the Spearman correlation coefficient.

## Results

Figure 1 shows the intra-operative changes in free radical activity as measured by the diene conjugated method. The mean base line plasma PMR was 10.87±0.78%, which although higher than those of healthy volunteers (9.74±0.84%), did not reach statistical significance. No significant change in activity occurred during the anhepatic phase (10.29±0.71%), but a marked rise in activity was evident following reperfusion with an initial peak at 1 h (24.42±7.81%, $p< 0.01$). A second more prominent peak appeared 12 h post-reperfusion following a transient decrease of radical activity (21.91±2.46%, $p<0.01$). Six patients had poor graft function according to the criteria defined earlier (Table 1) with scores ranging between 7 and 9. Of these, three had died on days 7, 12 and 31 days following acute post-transplant liver failure. Another patient from the same group developed features of PNF and required retransplantation on day 3. Fig. 2 shows the mean delta PMR activity, which is the difference between the maximum and base-line values plotted against the total score of graft outcome for each patient. A significant correlation was found: the higher the free radical activity, the worse the graft outcome ($p<0.01$).

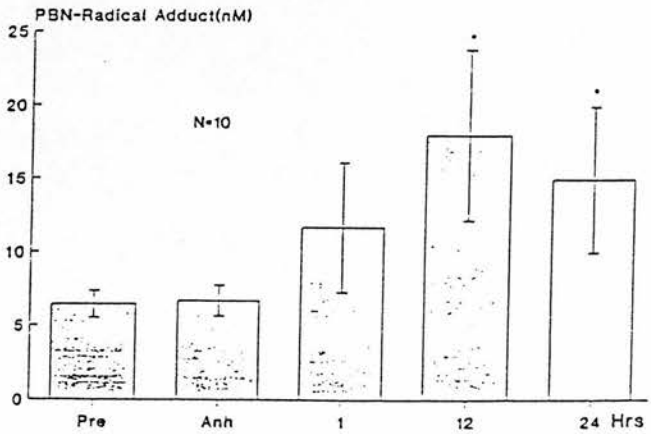


*Fig. 3. Peri-operative changes in free radical activity determined by EPR method. and identified as PBN-radical adduct signals. Pre: pre-transplant, Anh: during anhepatic phase, and then post-reperfusion at 1, 12, and 24 h.*

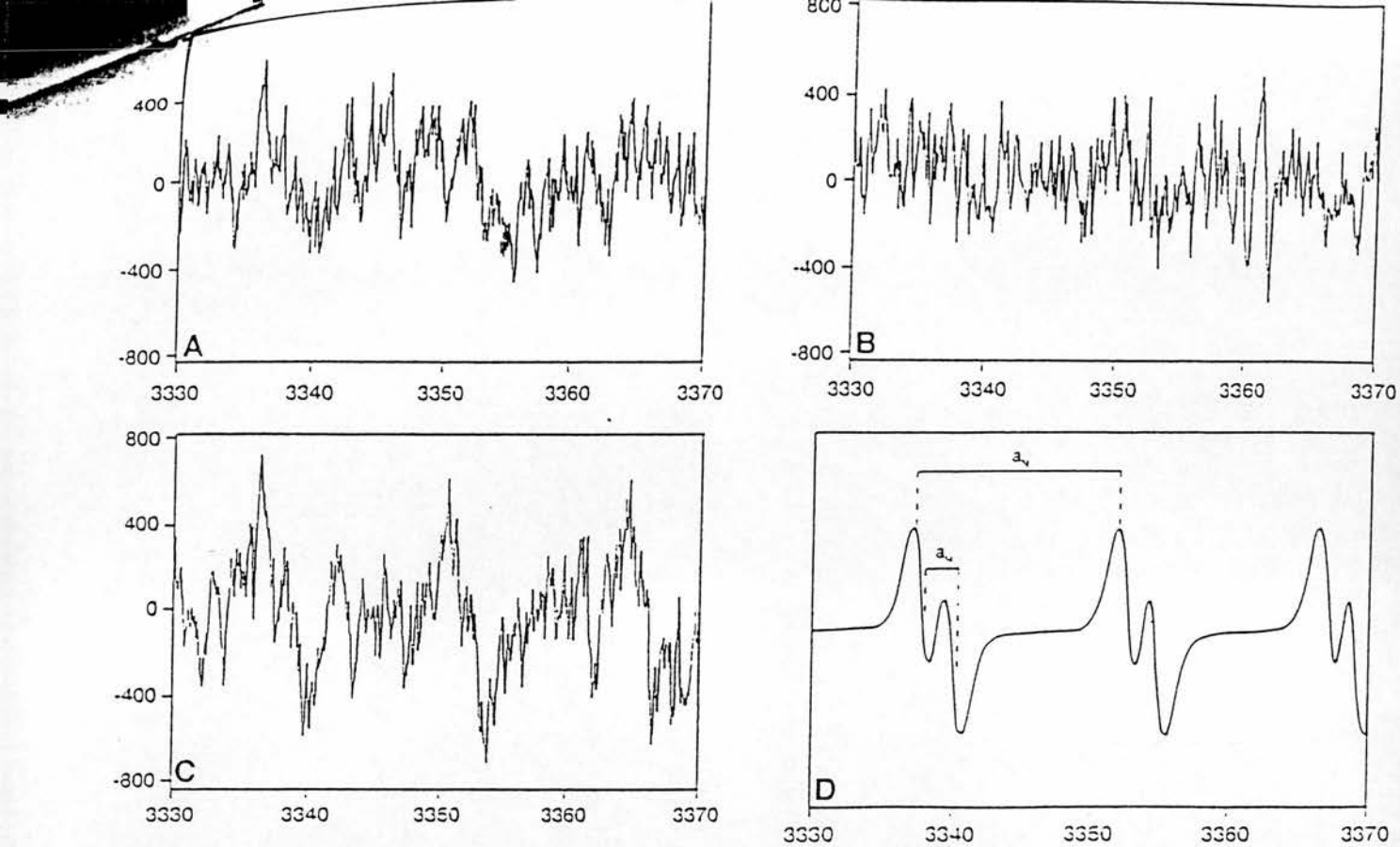


*Fig. 4. Representative time course of EPR spectra of PBN-radical adducts obtained from one patient: (A) prior to induction of anaesthesia, (B) during anhepatic phase, (C) 12 h after reperfusion and (D) spectral simulation of the PBN radical adduct, using the parameters $a_H$=0.19 mT, $a_N$=1.36 mT. Comparison of this simulation with the spectrum in (C), clearly shows the similarity of the radical species. Spectra (A) and (B) show evidence of the same radical, although at the lower concentration, the hydrogen hyperfine splitting is not resolved.*

Using the spin trapping technique, PBN-adduct signals were barely detectable after induction of anaesthesia and during the anhepatic phase (Fig. 3). A significant increase in intensity was evident following reperfusion with larger signals obtained in 12-h samples which showed 3 lines from a nitrogen atom, with $a_H$=14 gauss, and these are split into doublets by a hydrogen atom, with $a_N$=2 gauss. Such signals are identical to those reported by Tortolani et al. (13). Levels of PBN-radical adducts of those samples were 2–3 times higher than the pre-transplant levels ($p$<0.05). Representative EPR obtained from a patient before and after reperfusion of the graft are shown in Fig. 4.

There was no correlation between the magnitude of free radical changes and cold ischaemia time (10.61±0.58 h) or mean blood units transfused (7.27±1.12, range 2–19 units).

Acute rejection which required treatment, as judged by clinical and histological criteria, occurred in seven patients (30%). The mean PMR of patients with rejection was 11.69±0.84% compared with 11.17±1.54% in the non-rejection group (NS).

## Discussion

There is increasing evidence to suggest that oxidative stress, the result of excessive production of oxygen-derived and other radicals, is a major cause of reperfusion injury. These observations are based on experimental and animal model studies (7,14) and further supported by favourable effects of antioxidants such as allopurinol, ascorbic acid and superoxide dismutase when used in such situation (14,15).

In this study we have shown evidence of increased free radical activity following reperfusion of the new liver graft. We used two methods to quantitate free radical activity because of the limitations of the individual methods alone, as suggested previously (8). The PMR method is regarded as a "fingerprint" assay providing indirect evidence of radical production by

end-products of lipid peroxidation. in this case is diene conjugated isomer. These ve been the most frequently used methods for etecting free radical activity in human diseases for ieir safety, as they are primarily *in vitro* techniques. PR spectroscopy, on the other hand, is a direct and iantitative method which measures the energy ianges that occur as unpaired electrons align in sponse to an external magnetic field (8). As many ee radicals have a very short life (less than millisec-ids), such measurement therefore depends first on apping the radical by administering a compound ich as PBN (preferably intravenously) to produce a troxide (a spin-trapped adduct) whose lifetime is nsiderably longer than that of parent free radical. id therefore detectable by EPR spectroscopy (16). . human studies toxicity of PBN has limited the chnique to mixing of blood samples with the spin ap as soon as possible after collection. Despite the evitable reduction in the sensitivity of the technique ie to the time lag between free radical generation id trapping, it remains by far the most specific in etecting radicals, when compared with the other cur-ntly available techniques. Valuable data have been otained, for example, relating to free radical produc-on during angioplasty (17,18).

The results of this study have shown a significant egree of correlation between the degree of free radi-al activity and markers of graft function, which is uggestive of a role for free radicals in the aetiology f PGD. The criteria we used in this study (11) are a odification of those defined by Greig et al., in hich the widely accepted parameters for assessment f early post-operative graft function are used. They clude serum levels of aminotransferases, bile pro-uction and coagulation status, alone or in combina-on. In these criteria, a well-defined cut-off value for ach parameter is determined.

The currently available knowledge, however, does ot support the suggestion that reperfusion injury is mply a matter of damage to tissues by toxic radicals 9). Inflammatory mediators such as leukotrienes, AF, and soluble adhesion molecules (e.g. p-selectin) ave been shown to be upregulated by free radicals 0–22), and they have been implicated as well in perfusion injury (5,6,23). These molecules react ith the complementary ligands on the circulating eutrophils, leading to their arrest and consecutive tivation with release proteases and radicals (24,25). he biphasic appearance of free radical generation en in this study (Fig. 1) may support the view that ey are important in the initiation of the inflamma-ry cascade and consecutive reperfusion (26), as the earlier rise of free radicals is likely to result from xanthine oxidase on the endothelial cell surface, via the classic free-radical-mediated chain reaction with the late surge radicals being a direct product of activated neutrophils. It will be interesting to look into these aspects in more detail to determine the likely mechanisms of free radical generation at different time intervals. This would be of help in determining an appropriate therapeutic strategy to reduce reperfusion injury.

We did not see a correlation between cold ischaemia time and degree of free radical activity. Prolonged ischaemia can lead to increased accumulation of XO and hypoxanthine, which subsequently could determine the extent of free radical activity. It is possible that the limited ischaemia time in this study has been a factor, and this could be supported by findings of a previous study that showed extended preservation beyond 16 h in UW solutions is unfavourable and likely to influence subsequent graft function.

Acute graft rejection which required treatment occurred in 30% of the patients studied. We did not see a difference in free radical activity in the patients with rejection as compared with the non-rejection group. Poor graft function has been shown to increase the incidence of graft injection (3). The mechanism for such a relationship is, however, unclear, and from the results of this study it would seem unlikely to be free radical related.

In conclusion, this study provides evidence of increasing free activity following reperfusion of liver grafts and the magnitude of changes correlated with the severity of graft dysfunction.

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# NEUTROPHIL ELASTASE: A DETERMINANT OF ENDOTHELIAL DAMAGE AND REPERFUSION INJURY AFTER LIVER TRANSPLANTATION?

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**Reperfusion injury has been implicated in the development of primary graft dysfunction (PGD) after liver transplantation. Neutrophil migration and activation may be involved in the pathogenesis of this injury.**

**We studied neutrophil activation and its role in the etiology of PGD by measuring neutrophil elastase by radioimmunoassay, in serial blood samples of 19 patients before, during, and for 24 hr after transplantation. In a subgroup of patients, we also measured soluble thrombomodulin at the same time points as a marker of endothelial damage.**

**The pretransplant elastase level was significantly raised (40.13±4.84 ng/ml, mean ± SEM) compared with levels of healthy controls (18.7±5.6 ng/ml, $P<0.05$). A marked increase in elastase activity followed reperfusion, with a peak at 2 hr (370±50.5 ng/ml, $P<0.01$). Thereafter, there was a decline, but elastase remained elevated at 24 hr (186±60.94 ng/ml). The mean increase in neutrophil elastase after reperfusion correlated significantly with markers of graft function ($P<0.05$) and with the mean rise in soluble thrombomodulin**

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$P=0.042$), which increased from a pretransplant level of 81.2±11.32 to 186±50.4 ng/ml, 6 hr after reperfusion ($P<0.05$).

The results of this study indicate that marked neutrophil activation and endothelial cell damage occurs after graft reperfusion during orthotopic liver transplantation, and the degree of activation correlates with markers of graft function, which may suggest a role in the etiology of PGD.

After orthotopic liver transplantation (OLT*), many grafts show some degree of hepatitis, which when severe is associated with poor graft function, known as primary graft dysfunction (PGD). Patients with this complication have a greater risk of developing graft rejection (*1*).

Primary graft nonfunction (PNF) is an extreme form of PGD, occurring in about 10% of liver transplant cases. It is the most common indication for retransplantation within the first 2 weeks (*2*). Patients with PNF develop encephalopathy and hemodynamic instability, and have poor or no bile flow. Coagulation is markedly deranged in association with increased glucose requirements, raised transaminases, metabolic acidosis, and renal failure.

The pathogenesis of PNF remains unclear. Preservation and later reperfusion of the graft have been suggested to play a significant role in its etiology (*3*). We and others have produced evidence of increased free radical generation following reperfusion of a liver graft (*4–6*), probably by way of the classical hypoxanthine-xanthine oxidase pathway and from neutrophils. The latter have been shown in histopathological studies to accumulate in tissues with reperfusion injury (*7*); when their infiltration is prevented, using antineutrophil serum, monoclonal antibodies, or vinblastine pretreatment, ischemia-reperfusion damage is reduced (*8, 9*).

Activated neutrophils cause tissue damage by release of free radicals and proteolytic enzymes, such as metalloproteinases and elastase, from the granules. They are capable of direct tissue damage and enzymatic inactivation unless neutralized by their natural inhibitors (*10*). Neutrophil elastase (NE) is the most potent and, because of its specificity, has been established as a means of assessing in vivo neutrophil activation (*11*).

The endothelial cell damage by NE has recently been shown to result in cleavage and inactivation of thrombomodulin (TM), with the loss of transmembrane and cytoplasmic domains and release of the soluble form into the circulation. TM is a glycoprotein that acts as a receptor for thrombin on the endothelial cell surface. It plays an important role in maintaining the antithrombogenic property of the endothelial cell via the TM-thrombin-protein C complex (*12–26*).

In this study, we assessed the degree of proteolytic activity of neutrophils by determining the changes in NE before and after reperfusion in liver transplantation. We looked at the relationship of the changes with the parameters of subsequent graft function. Perioperative changes in soluble TM (sTM) were used as a marker of endothelial cell damage.

Abbreviations: NE, neutrophil elastase; OLT, orthotopic liver transplantation; PGD, primary graft dysfunction; PNF, primary graft nonfunction; sTM, soluble thrombomodulin; TM, thrombomodulin; VVB, venovenous bypass.

## MATERIALS AND METHODS

We studied 19 patients (7 men and 12 women; mean age, 56±4.5 years) who had undergone OLT for primary biliary cirrhosis (n=7), alcoholic liver disease (n=5), autoimmune hepatitis (n=4), primary sclerosing cholangitis (n=2), and subacute liver failure (n=1).

Blood was taken from an indwelling catheter in the right atrium before transplantation, during the anhepatic phase, and then after reperfusion at 0.5, 1, 2, 6, 9, 12, and 24 hr. Plasma was immediately separated by centrifugation (3000 rpm for 20 min) and stored at −40°C for later analysis.

*NE measurement.* Plasma NE was measured by radioimmunoassay as described previously (*13*). In brief, 50 μl of standard or sample was added to 50 μl of $^{125}$I-elastase (10 ng/ml) and 50 μl of antielastase antibody raised in rabbits and made up to a final volume of 200 μl with buffer (0.05 mol of phosphate [pH 7.4], 0.6 mol of NaCL, 2 mmol of EDTA, 20 U/ml heparin, 20 U/ml aprotinin, and 2% heat inactivated horse serum).

Samples were incubated at room temperature overnight and separated with donkey anti-rabbit immunoglobulin immobilized on Sepharose. After agitation at room temperature, the bound complex was separated from the free complex by sedimentation under gravity through a 10% sucrose solution, aspirated, and counted in a gamma-counter (NE 1600).

Plasma neutrophil elastase was expressed in ng/ml, and the sensitivity of the assay was 5 ng/ml with a 4% interassay coefficient of variation.

Results from the transplant patients were compared with those from healthy volunteers matched for age, as established in our laboratory reference ranges.

*Soluble TM measurement.* Plasma antigen levels of sTM were determined using an enzyme immunoassay, provided by Diagnostica stago, Asnieres, France, as described previously (*14*). In brief, a solid support (micro-ELISA plate) is coated with a mouse monoclonal antibody that binds sTM in the assay standard or in the patient's sample.

The bound sTM is next revealed by the use of a second mouse anti-sTM monoclonal antibody labeled with horseradish perioxidase, which binds to another antigenic determinant of the sTM molecule that is remote from the first one. The bound enzymatic activity is then demonstrated by its oxidative action on the substrate orthophenylene-diamine in the presence of hydrogen peroxide.

After the reaction has been stopped by the addition of sulfuric acid, the coloration that is obtained is measured at 492 nm. The observed optical density is directly proportional to the concentration of sTM. Results are expressed in ng/ml.

*Transplant details.* Donor age was 42±6 years. ABO blood group compatibility and matching of donor-recipient sizes were observed. As per protocol, grafts with macroscopic steatosis and grafts from donors with a known history of drug/alcohol abuse were rejected. University of Wisconsin solution was used for preservation.

All patients underwent venovenous bypass (VVB) during the anhepatic phase. It was started after the porta hepatis was dissected and the portal vein was clamped. The mean duration of VVB was 72±25 min. VVB was discontinued just before the portal vein was unclamped and the donor liver was reperfused.

Details of transfusion of blood and blood products perioperatively were recorded. After OLT, data collected included bile flow, serum transaminase levels (alanine aminotransferase), and prothrombin time. These were recorded daily up to the time of discharge, retransplantation, or death.

Early postoperative graft function was graded according to criteria described previously (*15*). Table 1 shows a scoring system dependent on serum alanine aminotransferase level and prothrombin activity determined 72 hr after surgery, and includes the mean value of bile output over the first 72 hr after transplantation. For each patient, the score was calculated from the sum of the assigned value for each parameter, ranging from 3 to 9. According to this criterion,

TABLE 1. Parameters of scoring system for assessment of early postoperative graft function in liver transplant patients (Ref. 15)

| Parameter | Assigned value |
|---|---|
| Serum ALT[a] (U/L) | |
| <1000 | 1 |
| 1000–2500 | 2 |
| >2500 | 3 |
| Bile output[b] (ml/day) | |
| >100 | 1 |
| 40–100 | 2 |
| <40 | 3 |
| Prothrombin activity[c] (%) | |
| >60 (spontaneous) | 1 |
| >60 (with fresh-frozen plasma) | 2 |
| <60 (despite fresh-frozen plasma) | 3 |

[a] Highest value in 72 hr after transplant. Normal value is up to 40 U/L. ALT, alanine aminotransferase.
[b] Mean value during the first 72 hr after transplant.
[c] Lowest value in the 72 hr after transplant.

patients with a total score of up to 6 (group I: up to 3; group II: 4–6) were shown to have good outcome. Scores of 7–9 (group III) were associated with poor outcome.

*Analysis.* Results are expressed as mean ± SEM. Statistical significance was accepted for $P<0.05$. The difference between the time points was calculated using one-way analysis of variance. The Tukey-HSD test (analysis of variance) was used to determine the significance of change in NE in relation to outcome. Spearman's rank correlation coefficient test was used to determine correlation.

## RESULTS

Before OLT, the mean NE level was 40.13±4.84 ng/ml, which was significantly higher than levels of normal controls (18.7±5.6 ng/ml, $P<0.05$). During the early anhepatic phase, NE levels increased moderately to 71.28±9.11 ng/ml ($P<0.05$). After unclamping of the portal vein (i.e., reperfusion), there was a marked increase in NE antigen, which peaked 2 hr after reperfusion (370±50.5 ng/ml, $P<0.001$). Although there was a gradual decline in the levels of NE, they remained elevated 24 hr after reperfusion (186±60.94 ng/ml), although this was not statistically significant (Fig. 1).

Two patients died of graft failure on days 7 and 12. Another patient died on day 31 after a stormy postoperative course,

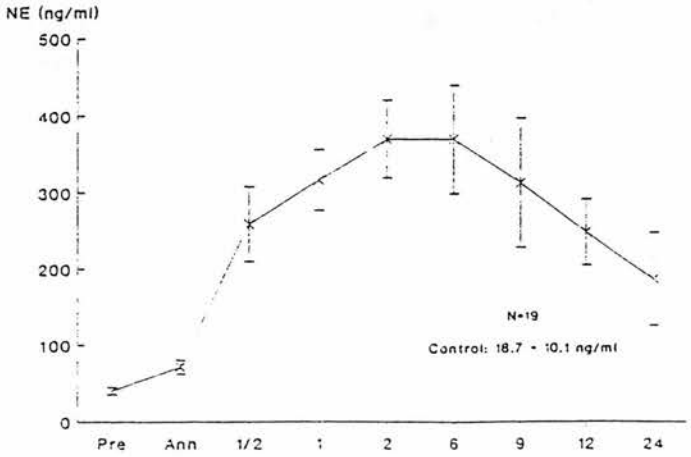


FIGURE 1. Changes in NE levels before and after reperfusion up to 24 hr. Pre, before induction of anesthesia; Anh, anhepatic phase.

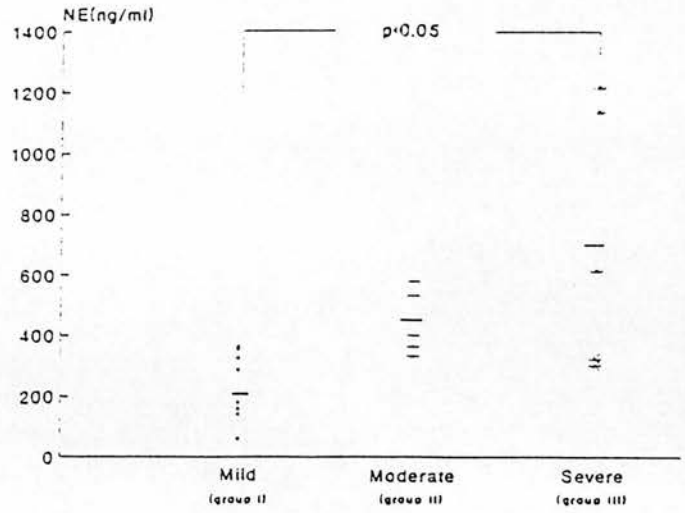


FIGURE 2. Changes in ΔNE in relation to graft outcome (patients classified into three groups according to their total outcome score, using the criteria in Table 1): group I, mild graft dysfunction, with a total score up to 3; group II, moderate dysfunction, with a score of 4–6; and group III, severe dysfunction, with a score of 7–9. A total score of up to 6 is usually associated with good outcome.

which started with initial poor graft function and was later complicated by acute rejection, septicemia, and massive gastrointestinal bleeding due to multiple cytomegalovirus-induced ulcers involving different parts of the gastrointestinal tract. The outcome scores of these patients ranged between 6 and 8.

Mean delta NE (the difference between maximum and baseline values for each patient) was 224.8±36.3 ng/ml in group I, 442.6±48.5 ng/ml in group II, and 720±195.8 ng/ml in group III. Using analysis of variance (Tukey-HSD test), we found that only patients of group III showed a significant increase in NE, as compared with group I patients ($P<0.05$), Fig. 2.

The mean baseline level of sTM was 81.2±11.32 ng/ml (Fig. 3). It increased during the anhepatic phase to 88.5±29.8 ng/ml; however, this did not reach statistical significance. After reperfusion, there was a marked increase in sTM, which reached a maximum mean value of 186±50.4 ng/ml at

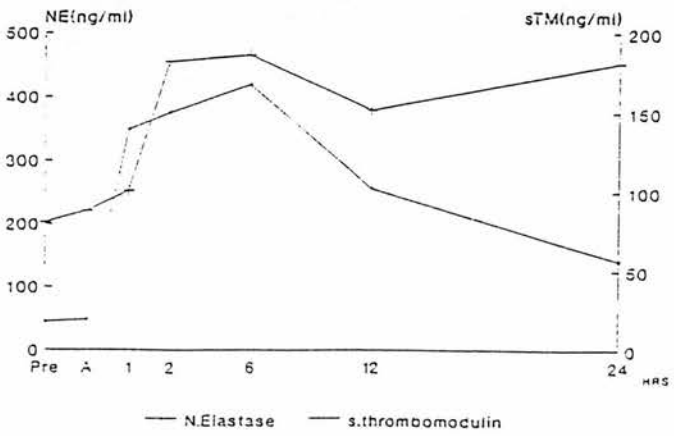


FIGURE 3. Changes in sTM and NE levels before transplantation (Pre), during the anhepatic phase (A), and after reperfusion up to 24 hr.

; hr after reperfusion ($P<0.05$). The mean postreperfusion crease of sTM correlated with that of NE ($P=0.042$).

Mean requirements were: blood transfusions, 8.07±7.47 U ange, 2–24 U); platelets, 5.47±1.36 U (range, 0–20 U); yoprecipitates, 4.2±1.88 U (range, 0–20 U), and fresh-ozen plasma, 8.27±1.01 U (range, 2–15 U). There was no gnificant difference in any of the above parameters in the ıtients of the different groups. No significant correlation as found between these parameters and the changes in NE · sTM.

## DISCUSSION

Histological studies demonstrating infiltration of neutro-hils in tissues that had been exposed to transient ischemia ollowed by reperfusion support the notion that neutrophils ontribute to reperfusion injury (*7*). Once released from neu-rophils, NE causes direct damage to intact cells and inacti-ates various proteins, such as immunoglobulins, comple-ents, and clotting factors (*10*). Furthermore, NE facilitates ncreased generation of free radicals by converting xanthine ehydrogenase into oxidase in the vascular endothelial cells *6*). Such an effect would set the scene for a vicious cycle by hich free radicals, in addition to their direct tissue damage, ould contribute to further neutrophil chemoattraction, roll-ng, and adhesion via enhancing expression of pro-inflamma-ory mediators such as adhesion molecules (e.g., GMP-140), latelet-activating factor, and the leukotriene LTB4 (*17–19*). ree radicals also inhibit neutralizing NE by its specific nhibitor $\alpha_1$-antitrypsin via oxidation of methionine at posi-on Met 358 (*20*).

We have shown that the baseline values of NE in the atients studied were higher than those of a healthy volun-er population. This is in keeping with the observations of revious studies from our group, and others that demon-rated evidence of neutrophil activation in patients with ronic liver diseases, particularly those with an alcoholic iology (*21*, *22*).

The level of NE increased markedly after reperfusion and as sustained for several hours thereafter. The relatively hort duration between the samples taken during the anhe-atic phase and samples from the early postreperfusion pe-od further suggests that neutrophil activation, rather than, r instance, delayed renal or hepatic clearance, is the likely echanism of such changes.

Changes in NE activity after reperfusion were significantly gher in patients with poor graft function, using the criteria escribed in Table 1. Patients in group II showed higher ean NE levels compared with group I patients; however, e changes did not reach statistical significance. The high-t values were seen in two of the three patients who died ter surgery as a result of poor graft function. Such findings rther suggest that neutrophils play a significant role in the iology of reperfusion injury.

The degree of rise in NE levels following reperfusion seen this study was markedly higher than that detected in nditions such as acute myocardial infarction, multiple auma, or inflammatory bowel diseases (*13*, *23*, *24*). This ight indicate more significant neutrophil-induced damage llowing liver graft reperfusion when compared with the ove-mentioned conditions. The fact that we collected our mples from a site more central and closer to the likely site of increased activity could have played a significant role in such differences.

Although VVB could potentially cause neutrophil activation, we do not believe that VVB played a significant role in the changes seen in this study, as the most marked increase in NE levels occurred well after VVB was discontinued and remained so for several hours thereafter. In some patients, significantly high levels of NE were seen 24 hr after discontinuation of VVB. In addition, the continuous infusion of aprotinin, which inhibits platelet aggregation and formation of NE-$\alpha$1 antitrypsin complex during VVB, reduces neutrophil activation further (*25*).

In this study, sTM levels increased significantly after reperfusion, which is comparable to the results of a recent study in patients who had OLT (*26*). Mean perioperative changes of sTM correlated with that of NE, which may further support the hypothesis that release of sTM from endothelial cells into the circulation is a consequence of neutrophil activation.

Neutrophilic proteases were shown to decrease TM activity of the endothelial cell membrane by a specific hydrogen peroxide ($H_2O_2$) facilitating mechanism. This is thought to be oxidation of Met 388 in the TM molecule (*27*). Loss of the anticoagulant property of the endothelial surface in such a mechanism potentially leads to microcirculatory stagnation and tissue ischemia, and this might explain the onset of the no-reflow phenomenon, a recognized feature of preservation-reperfusion injury.

In conclusion, this study demonstrates a significant degree of neutrophil activation following graft reperfusion during OLT, with evidence of endothelial cell damage as a result of such activity. The degree of activation correlated with the severity of PGD, which may suggest a role for neutrophils in its etiology. Further work is required to understand the important factors that determine neutrophil chemoattraction and activation following reperfusion. This will help in developing strategies to prevent such damage and improve graft function.

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# RELATIONSHIP OF FK506 WHOLE BLOOD CONCENTRATIONS AND EFFICACY AND TOXICITY AFTER LIVER AND KIDNEY TRANSPLANTATION

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**FK506 (tacrolimus) is a safe and effective immunosuppressant for the prevention of organ rejection after organ transplantation. FK506 has a relatively narrow therapeutic index and the correlation of dose to blood concentration is poor as a result of moderate variability in pharmacokinetic parameters between patients. Therapeutic monitoring of whole blood FK506 drug concentrations has been used in an effort to determine whether a relationship exists between concentrations of FK506 in the blood and the development of toxicity or the risk for organ rejection. An analysis of the relationship between FK506 blood levels and the occurrence of toxicity and rejection was carried out using data from four recent clinical trials. Trough FK506 levels within a 7-day window before the onset of rejection or toxicity were analyzed using logistic regression models. In kidney transplant patients (n=92), a significant correlation between FK506 levels and the incidence of both toxicity ($P=0.01$) and rejection ($P=0.02$) was seen. In liver transplant patients from three clinical trials, FK506 levels correlated well**

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# INFLUENCE OF CYCLIC GUANOSINE MONOPHOSPHATE CHANGES ON HEMODYNAMICS AFTER REPERFUSION IN LIVER TRANSPLANTATION

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**Orthotopic liver transplantation (OLT) is often associated with hemodynamic instability upon reperfusion, recognized as postreperfusion syndrome. Changes in vascular tone due to humoral factors released upon reperfusion of the graft have been suggested as a possible mechanism. In this study, we looked at the perioperative changes in cyclic guanosine monophosphate (cGMP), a mediator of vascular smooth muscle relaxation, and investigated its relationship with hemodynamic parameters.**

**cGMP was measured in the plasma of 14 patients undergoing OLT by radioimmunoassay serially at the preanhepatic and anhepatic phases, and after reperfusion at 30, 60, and 120 min. Hemodynamic data recorded were systemic and pulmonary arterial pressures, cardiac output, and pulmonary and systemic vascular resistance.**

**cGMP decreased markedly after reperfusion from a baseline level of 5.33±0.7 ng/ml to 1.63±0.5 ng/ml ($P<0.01$). Pulmonary arterial pressure increased from 17±1.21 mmHg to 23.5±1.9 mmHg ($P<0.05$), and pulmonary vascular resistance increased from 62.8±12.9 dynes/sec/cm$^5$ to 135±42.7 dynes/sec/cm$^5$ ($P<0.01$). Changes in cardiac output and systemic vascular resistance were not significant. The changes in cGMP correlated with pulmonary arterial pressure (r=0.74, $P=0.005$) and pulmonary vascular resistance (r=0.7, $P=0.01$).**

**These data confirm the occurrence of hemodynamic changes during OLT, and provide evidence to suggest that the reduction in cGMP after reperfusion may mediate the vascular changes.**

The reperfusion phase during orthotopic liver transplantation (OLT*) is a critical event with well-recognized and sometimes profound hemodynamic and cardiac changes that may be responsible for intraoperative death (*1*). The hemodynamic changes, defined as postreperfusion syndrome, are predominantly an increase in pulmonary capillary wedge pressure (PCWP) and central venous pressure (CVP), along with a reduction in mean arterial pressure (MAP) and systemic vascular resistance. Such hemodynamic derangement tends to improve in the majority of patients within 30–60 min, requiring only cardiovascular pharmacological support. However, the changes may be severe enough in a small proportion of patients to be life threatening (*2*).

Several potential mechanisms for these changes have been considered, including sudden influx of cold, acidic and hyperkalemic blood, embolization, and release of vasoactive substances from the grafted liver (*3*). The ischemia and reperfusion that accompany organ transplantation are asso-

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* Abbreviations: cGMP, cyclic guanosine monophosphate; CO, cardiac output; CVP, central venous pressure; MAP, mean arterial pressure; NO, nitric oxide; OLT, orthotopic liver transplantation; PAP, pulmonary arterial pressure; PCWP, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance; SVR, systemic vascular resistance.

ciated with release of proinflammatory mediators, generation of free radicals, and neutrophil sequestration and activation (*4*). Such mediators could influence endothelial cell function, and lead to disturbance of vasomotor control. Free radicals have been shown to counteract the relaxation effects of nitric oxide (NO) on vascular smooth muscle cells via inhibition of guanylyl cyclase and reduction in the release of cyclic guanosine 3′,5′-monophosphate (cGMP) from endothelial cells (*5*).

This study was designed to test the hypothesis that the hemodynamic changes associated with reperfusion may be due to alteration in cGMP activity.

## PATIENTS AND METHODS

*Patient details.* Fourteen patients (six men and eight women) with a mean age of 52.4±5.4 years were studied. The indication for transplantation was primary biliary cirrhosis (n=6), alcoholic liver disease (n=5), and cryptogenic cirrhosis (n=3). All patients underwent venovenous bypass during the anhepatic phase of the transplant procedure, with a mean duration of the anhepatic phase of 71.5±9.3 min.

Detailed hemodynamic measurements were recorded after induction of anesthesia, during the anhepatic phase, and after reperfusion at 30, 60, and 120 min. Pulmonary arterial pressure (PAP) and PCWP were measured using a Swan-Ganz catheter, and MAP was measured continuously using an intra-arterial line. Cardiac output (CO) measurements were obtained in triplicate using the thermodilution technique. Systemic vascular resistance (SVR) and pulmonary vascular resistance (PVR) was calculated using the standard equations: $SVR$ (dynes/sec/cm$^5$) = $(MAP - CVP) \times 79.9/CO$, and $PVR$ (dynes/sec/cm$^5$) = $(PAP - PCWP) \times 79.9/CO$.

We also recorded the amount of fluid administered to maintain hemodynamic stability, and the blood products used to maintain hemoglobin concentration >10 g/dl, platelet count >$50\times10^9$/L, and prothrombin time <16 sec.

*Measurement of cGMP.* Blood samples were collected from a catheter placed at the right atrium at the same time points as the hemodynamic recordings. Plasma was separated immediately (centrifuged for 15 min at 3000×*g*) and stored at −40°C until analysis.

cGMP was measured as described previously (*6*). Standards and samples were assayed in duplicate. The standard was prepared by double dilution of a stock of 32 nmol/L solution of cGMP to yield a concentration gradient from 0.03125–32 nmol/L. One hundred microliters of each concentration were assayed as described below for the samples.

Plasma (500 μl) was extracted into 1 ml of ethanol. The ethanolic fraction was dried under air, and the pellet was resuspended in 500 μl of assay buffer. Fifteen microliters of acetylation mixture were added to 100 μl of the assay buffer and combined with 150 μl of $I^{125}$-cGMP (diluted to give 400 cpm/150 μl) and 100 μl of rabbit anti-human cGMP polyclonal antibody (diluted to give 50–60% binding). After thorough mixing, the samples were allowed to equilibrate for 18 to 24 hr at 4°C. After equilibration, bound and unbound $I^{125}$-cGMP were separated by the addition of 600 μl of dextran-coa charcoal and spinning (3000 rpm, 15 min, 4°C). The aqueous la was removed and the bound trace was measured on a beta-count The coefficient of variation for the assay was 3.5%, and the int assay variation was 4.3%. Results are expressed as ng/ml.

*Statistical analysis.* All results were expressed as mean ± S The difference between mean before and after reperfusion was d termined by one-way analysis of variance. Correlation between va ables was determined using Spearman's rank correlation coefficie Values were considered statistically significant when the *P*-val was less than 0.05.

## RESULTS

Changes in the hemodynamic parameters are summariz in Table 1. The main changes were of PVR and PAP. The PV increased slightly during the anhepatic phase from a bas line value of 62.8±12.9 dynes/sec/cm$^5$ to 72.5±9.6 dynes/s cm$^5$ (NS). More marked changes were seen after reperfusio PVR increased to 135±42.7 dynes/sec/cm$^5$ ($P<0.01$), and PA increased from 17±1.2 mmHg to 22.8±2.6 mmHg ($P<0.0$ 30 min after reperfusion. The latter increased to a maximu of 23.5±1.9 mmHg 2 hr after reperfusion.

CO decreased slightly but not significantly during the a hepatic phase to 8.8±0.78 L/min from a mean pretranspla value of 10.3±2.4 L/min. MAP decreased significantly at min after reperfusion ($P<0.05$), but improved spontaneous and subsequently returned to baseline values.

The mean pretransplant level of cGMP was 5.33±0.7 ng/ (Fig. 1), which was significantly higher than the levels healthy volunteers (1.35±0.43 ng/ml, n=10, $P<0.01$). cGM increased marginally during the anhepatic phase 6.31±0.76 ng/ml (NS), but after reperfusion there was rapid and significant decrease in cGMP to 1.63±0.50 ng/ ($P<0.01$).

The baseline cGMP correlated inversely with SVR a MAP ($P<0.05$ and $P=0.023$, respectively) and directly w PVR recorded at the same time point ($P<0.05$). The corre tion, however, was more obvious between the mean chang in postreperfusion cGMP and those of PVR (r=0.7, $P=0.$ n=12) and PAP (r=0.74, $P=0.005$, n=12) (Figs. 2 and 3). correlation was detected between the intraoperative cGM changes and those of CVP and CO at any time point.

Requirements were: mean blood transfusion, 7.3±4.2 (range, 2–18 U); cryoprecipitates, 3.6±1.5 U (0–16 U); pla lets, 5.2±1.3 U (0–10 U); and fresh frozen plasma, 7.5±2.1 (2–12 U). No correlation was found between these paran ters and the changes in cGMP.

All patients received dopamine at a fixed infusion rate o μg/kg/min. Seven patients required noradrenaline (8 mg/1 ml) at a rate of 4.5±1.2 ml/hr, and four of these patients a

TABLE 1. Changes in hemodynamic parameters during OLT

| Time | MAP (mmHg) | CVP (mmHg) | CO (L/min) | SVR (dynes/sec/cm$^5$) | PAP (mmHg) | PVR (dynes/sec/cm$^5$) |
|---|---|---|---|---|---|---|
| Preanhepatic | 88.5±1.67 | 6±0.6 | 10.3±1.7 | 633±106 | 17±1.21 | 62.8±12.9 |
| Anhepatic | 87±2.07 | 6.5±0.86 | 8.8±0.78 | 731±67.8 | 16.8±0.92 | 72.5±9.6 |
| Postreperfusion | | | | | | |
| 30 min | 74±2.47 | 6.2±0.9 | 9.6±1.3 | 571±76 | 22.8±2.6[a] | 135±42.7[b] |
| 60 min | 97±51.75 | 7.5±0.7 | 9.3±1.29 | 612±61.3 | 23.1±2.4[a] | 111.7±30.2[a] |
| 120 min | 82±2.13 | 8.5±0.6 | 8.8±1.0 | 660±66.6 | 23.5±1.9[a] | 106.8±14.6[a] |

[a] $P<0.05$.
[b] $P<0.01$.

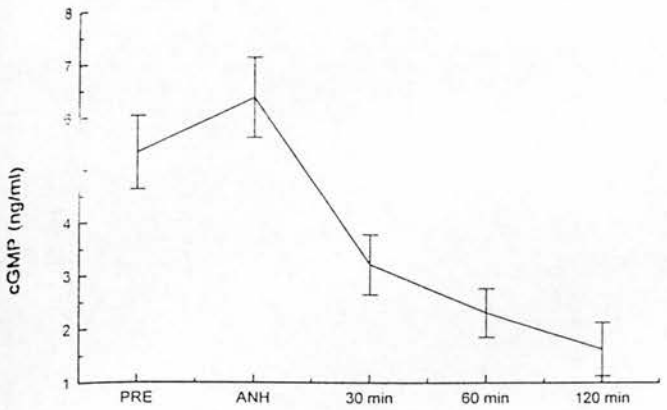


FIGURE 1. The perioperative profile of cGMP (PRE = preanhepatic, ANH = anhepatic, and 30, 60, and 120 min = postreperfusion time points). Control: 1.35±0.43 ng/ml.

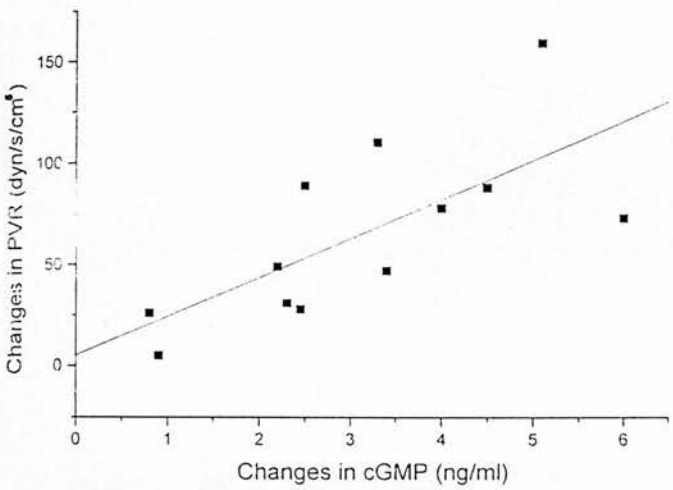


FIGURE 2. Correlation of the changes in cGMP (difference between pretransplant value and minimum postreperfusion one for each patient) with those of PVR (r=0.7, $P$=0.01).

required adrenaline (1:100,000) at a rate of 3±0.5 ml/hr. The mean postreperfusion change in cGMP was 2.9±0.7 ng/ml in these patients and 2.7±0.65 ng/ml in patients who did not require any support (NS).

## DISCUSSION

This study confirms the previous observations of major hemodynamic disturbances during OLT (*7*). The most striking changes were in PVR and PAP, both of which rose shortly after reperfusion. The changes in PVR and PAP correlated with the changes in cGMP levels.

cGMP, the vascular smooth muscle cell relaxant (*5*), was elevated in this group of patients with end-stage chronic liver disease prior to transplantation. Reduction in the concentration of cGMP after reperfusion could be a result of decreased activity of its main stimulants, or perhaps it could represent mere normalization. Whatever the mechanism behind such changes, the reduction in cGMP levels after reperfusion of the graft and the correlation with the changes in the pulmonary hemodynamic parameters suggest a causal relationship.

Although levels of cGMP continued to fall after reperfusion, PVR showed signs of recovery. There is no direct explanation for these changes. One possibility could be the release of other vasomotor mediators that may interfere with the actions of cGMP. Prolongation of the hemodynamic readings and measurements of cGMP for several hours afterward may help delineate this relationship further.

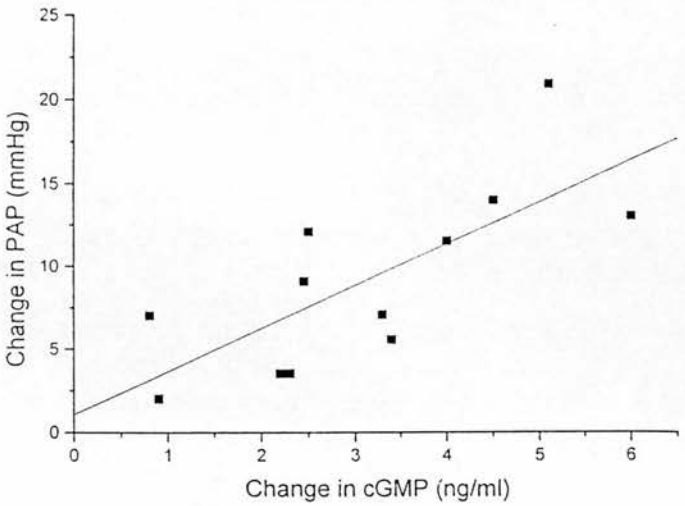


FIGURE 3. Correlation of the changes in cGMP with the those of PAP (r=0.74, $P$=0.005).

Our finding of elevated pretransplant cGMP levels is comparable to previous reports of elevated levels of NO, the main stimulant of cGMP generation in patients with cirrhosis (*8, 9*). Niederberger et al. (*10*) have shown in a cirrhotic animal model an increase in aortic cGMP that was reduced significantly after inhibition of NO production by the NO synthase inhibitor $N^G$-monomethyl-L-arginine. This reduction in NO was followed by reversal of the hyperdynamic abnormalities of the systemic circulation. Recently, Sogni et al. (*11*) showed a significant correlation between endogenous NO and the pulmonary vasodilatation seen in cirrhosis. Addition of the cGMP analog 8-(4-chlorophenylthio)-guanosine-3′,5′-cGMP to the preservation fluid in an experimental rat model of lung transplantation led to a significant reduction of PVR and an increase in pulmonary artery blood flow after reperfusion (*12*). These findings further support our conclusion about the role of cGMP in the hemodynamic alterations observed during transplantation.

Although cGMP may be generated by pathways that do not involve NO, such as stimulation of membrane-associated guanylyl cyclase by atrial natriuretic peptide or by enterotoxins, NO is the major stimulant for cGMP release from the soluble guanylyl cyclase (*13*). cGMP is released by virtually all cell types through activation of guanylyl cyclase. The levels of cGMP are regulated by its rate of synthesis by guanylyl cyclase and its rate of hydrolysis by cyclic nucleotide phosphodiesterase (*14*), and its half-life ranges between 10 and 20 min (*15*).

The release and effects of cGMP and NO could be influenced by free radicals, which are generated after ischemia-reperfusion. NO is the only known biological molecule that can be produced in high enough concentrations to outcompete superoxide dismutase for radicals. This interaction results in formation of peroxynitrite anion ($ONOO^-$), a radical with vasomotor properties unlike those of its parent compound

NO (*16*). Superoxide anion has been shown to induce vasoconstriction, and is known to have a direct inhibitory effect on guanylyl cyclase that leads to a decrease in cGMP production (*5*). It is plausible to consider that free radicals could play a role in the changes of cGMP observed in this study.

In summary, this study demonstrates hemodynamic changes affecting predominantly the pulmonary vasculature after reperfusion of liver graft during OLT. These changes correlated significantly with a reduction in the levels of cGMP, the mediator of vascular smooth muscle relaxation, which indicates a causal relationship. Further studies looking at the relationship of cGMP and NO with the other mediators that rise after graft reperfusion will be of major interest. These will provide the pathophysiological basis for therapeutic options of maintaining cardiovascular stability during the critical phase of reperfusion in organ transplantation.

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